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PURIFICATION AND CHARACTERISATION OF A STEROID-INDUCED MODULATOR OF NEUTROPHIL FUNCTION

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A thesis submitted for the degree of Doctor of Philosophy (Ph.D.) to
Division of Infection and Immunity,
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Declaration

Except where otherwise stated, the work presented in this thesis is original, and has been carried

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Dedication

*To my wife Pam
and my children Robyn and Jonathan*

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List of Abbreviations

AA	Arachidonic acid
AU	Absorbance units
AUFS	Absorbance unit full scale
BAE	Bovine aorta endothelial
BSA	Bovine Serum Albumin
BSS	Balanced Salt Solution
Ca ²⁺	Calcium ion
cAMP	cyclic Adenosine Monophosphate
CD	Cluster of Differentiation
cGMP	cyclic Guanosine Monophosphate
CM	Culture medium
CMS	Control Monocyte Supernatant
CO ₂	carbon dioxide
Cox	Cyclo-oxygenase
CRF	Corticotropin-releasing factors
Da	Dalton
DMEM	Dulbecco's modified eagles medium
DNDH	7-dimethylamino-napthalene-1,2-dicarboxylic acid hydrazide
F10	Ham's F10 Culture medium
EDTA	di sodium ethylenediaminetetra acetate
EGTA	Ethyleneglycol-bis-(α -aminoethyl ether) N,N,N',N'-tetraacetic acid
Fig	Figure
fMLP	n-formyl-methionyl-leucyl-phenylalanine
g	gram
GM-CSF	Granulocyte macrophage colony stimulating factor
GTP	Guanosine Triphosphate
H ₂ O ₂	Hydrogen Peroxide
HEPES	
HETE	Hydroxyeicosatetraenoic Acid
HH	Hank's HEPES
HO	HEPES Water
HPLC	High Performance Liquid Chromatography
HS	HEPES Saline
HUVEC	Human Umbilical Cord Endothelial Cells
ICAM	Intercellular Adhesion Molecule

Contents

IL	Interleukin
INF γ	Interferon gamma
iNOS	inducible nitric oxide synthetase
kDa	kilo daltons
L	Litre
LAD	leucocyte adhesion deficiency
LC-1	Lipocortin-1
LPA	lysophosphatidic acid
LPS	Lipopolysaccharide
LT	Leukotriene
M	Molar
Maldetof	Matrix assisted laser desorption time of flight
MCP-1	Monocyte chemotactic protein-1
MEM-S	Modified eagle's medium + 10% foetal bovine serum
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
MML	Mixed mononuclear leucocytes
MPa	Megapascal
MW	Molecular weight
$^{51}\text{NaCr}$	Radioactive Sodium chromate
NF κ B	Nuclear factor kappa B
nm	nanometre
NO	Nitric oxide
NSAID	Non-Steroidal anti-inflammatory Drug
NT	Neurotensin
O $_2^-$	Superoxide Ion
PAF	platelet activating factor
PBS	Phosphate Buffered Saline
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PG	Prostaglandin
PIP $_2$	Phosphatidyl Inositol bis Phosphate
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate

Contents

PMN	Polymorphonuclear Leukocyte
PMS	Polymorph Migration Stimulator
rcf	relative centrifugal force
rpm	revolutions per minute
STMS	Steroid Treated Monocyte Supernatant
T β 4	Thymosin β 4
T β 4ox	Thymosin β 4 with an oxidised methionine residue
TFA	Trifluoroacetic acid
TNF α	Tumour Necrosis Factor alpha
TX	Thromboxane
μ Ci	micro curie
μ g	micro gram
μ l	micro litre
μ m	micron
μ M	micro molar
VCAM-1	Vascular cell adhesion molecule-1
VIP	Vasoactive intestinal peptide
V _o	Volume of elution of excluded molecules
V _t	Volume of elution of included molecules
v/v	Volume by volume
w/v	Weight by volume

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1. Chettibi, S., Lawrence, A.J., Stevenson, R.D., and **Young, J.D.** (1993). Effect of lysophosphatidic acid on motility, polarisation and metabolic burst of human neutrophils. *FEMS, Immunology and medical microbiology*, **8**: 271-282.
2. Chettibi, S., Lawrence, A.J., **Young, J.D.**, Lawrence, P.D. and Stevenson, R.D. (1994). Dispersive locomotion of human neutrophils in response to a steroid-induced factor from monocytes. *Journal of Cell Science*, **107**: 3173-3181.
3. **Young, J.D.**, MacLean, A.G., Lawrence, A.J., Stevenson R.D. and Chettibi, S. (1997). Relationship of human neutrophil morphology and actin distribution to dispersive locomotion caused by a steroid induced factor. *EBO online*,

Web page:

<http://link.springer.de/link/service/journals/00898/fpapers/7002001/70020007.htm>

Aims of the project

The search for an effective anti-inflammatory therapy for treatment of debilitating chronic inflammatory diseases was thought to be over when glucocorticoid steroids were prescribed for sufferers of rheumatoid arthritis. The improvement in the condition of patients was dramatic, but the unwanted side-effects have meant the use of glucocorticoids as anti-inflammatory agents has been limited.

Stevenson, 1978 described a potential second messenger of the anti-inflammatory actions of these steroids in his M.D. thesis, secreted by cultured monocytes in the presence of steroids.

The main aim of this project was the purification of the factor(s), present in the supernatant of these cultured monocytes (STMS), responsible for the effect on neutrophil motility described by Chettibi et al., 1993.

In order to fulfil this aim it was necessary to develop the cell tracking assay data analysis by utilising the data produced from a program written in C by P.D. Lawrence. This required the writing of a procedure in C (Appendix I) that processed raw data from cell tracking experiments and calculated the parameters of cell locomotion described by Gail and Boone, 1970, 1972. This assay allowed the comparison of the behaviour of neutrophils in response to various stimulators of neutrophil motility.

The unusual way in which STMS-treated cells behave in this tracking assay i.e. high diffusion coefficient and persistence in a uniform concentration, provided parameters for the identification of active samples during the purification protocol.

It was also of interest to study the effects of STMS on other neutrophil functions, initially to understand the nature of the persistent locomotion observed. F-actin content of neutrophils treated with STMS, and a novel tilt assay was utilised for this purpose.

A number of experiments designed to assess the potential anti-inflammatory actions of the active factor(s) in STMS were also performed.

The adhesion of neutrophils to endothelial cells is a crucial step in the inflammatory response of these cells, and so their adhesion to bovine aorta endothelial cells was assayed under the influence of STMS.

Chemotaxis of neutrophils in response to a gradient of pro-inflammatory mediators is also an important stage in the inflammatory response. The effect of STMS on neutrophil chemotaxis in response to fMLP was assayed using a modified Boyden chamber.

The release of lysophosphatidic acid (LPA) by thrombin-activated platelets at physiologically relevant concentrations (Eichholtz et al., 1993) led to the assaying of various concentrations of LPA in the neutrophil tracking assay. The surprising findings that neutrophils responded to LPA by altering shape and increasing motility led to the study of the effect of LPA on the fMLP and PMA-induced metabolic burst of neutrophils using DNDH-dependent chemiluminescence. The potential role of LPA as a modulator of neutrophil function *in vivo* is discussed.

SUMMARY

A monocyte-derived factor that stimulates the locomotion of human neutrophils on an albumin-coated glass surface has been prepared from the culture supernatant of dexamethasone-treated human monocytes and called STMS (steroid-treated monocyte supernatant). A modified cell tracking program has been developed and the parameters of locomotion determined by the analysis of Gail and Boone (1970, 1972) for cells moving in a persistent random walk. Cells moving in uniform concentrations of STMS, interleukin-8 (IL-8) and n-formyl-methionyl-leucyl-phenylalanine (fMLP) chosen to give a sub-maximal speed of locomotion show persistent, random and constrained random diffusion respectively with augmented diffusion coefficients of 0.8 ± 0.1 , 0.14 ± 0.02 and $0.12 \pm 0.03 \mu\text{m}^2$ per second for STMS, IL-8 and fMLP respectively. The augmented diffusion coefficient and the underlying persistence are therefore sensitive quantitative assay parameters for STMS activity and the qualitative characteristics of locomotion allow STMS activity to be distinguished from that of all other factors tested. The contribution of lowered adhesion to locomotion was examined in a novel tilt-assay which demonstrated that cells in the presence of STMS, but not other factors, moved down slope with significantly increased speed while maintaining contact with the substratum. The results were interpreted in terms of the bipolar form of STMS-treated cells, contrasting with multipolar forms in response to other agents. This together with low adhesiveness plus an inherent tendency of a single locomotor focus to continue motion in its original direction has been used to explain the difference between response to STMS and other factors. STMS has been proposed to prevent directed locomotion of neutrophils to an

inflammatory site or to promote dispersive locomotion away from such a site, and perhaps to inhibit neutrophil transmigration between endothelial cells.

STMS also inhibits adhesion of neutrophils to bovine aorta and human endothelial cells by a yet undetermined mechanism. It induces unique changes in neutrophil shape with a characteristic monopole of F-actin distribution, which may correlate with the dispersive locomotion observed in the absence of a concentration gradient. This factor also inhibits n-formyl-methionyl-leucyl-phenylalanine-induced chemotaxis of neutrophils in a modified Boyden chamber assay. The reduction of adhesion and the inhibition of chemotaxis by the factor *in vitro* indicate a possible *in vivo* anti-inflammatory role.

The dispersive locomotion of neutrophils induced by this factor was utilised to monitor the purification of the active molecule, using standard chromatography techniques, present in the starting monocyte supernatant. The purification procedure identified a derivative of Thymosin β 4 with an oxidised methionine residue (T β 4ox) as the molecule responsible for the dispersive locomotion of neutrophils on the cell tracking assay. The shift in the elution of thymosin β 4 from a reversed-phase chromatography column following oxidation by hydrogen peroxide was utilised to confirm the activity was in fact associated with T β 4ox and not a contaminant. Thymosin β 4 was purified from human neutrophils using a reversed-phase chromatography column, 50% oxidised with hydrogen peroxide, and re-applied to the same column. The shift in elution meant that any contaminants present in the thymosin β 4 would be removed. Assays of this sample and the non-oxidised thymosin β 4 have confirmed the dispersive locomotion of human neutrophils in the cell tracking assay by STMS is caused by the presence of T β 4ox.

Parallel purification of thymosin β 4 from control monocyte supernatants (CMS) and STMS have indicated that thymosin T β 4ox is present in greater quantity in STMS, confirming the steroid-induction of its release into the culture supernatant. These results indicate that thymosin β 4, with an oxidised methionine residue, secreted by monocytes and macrophages, could be an important second messenger in the anti-inflammatory actions of glucocorticoids.

During the investigation into mediators of neutrophil functions, particularly locomotion, lysophosphatidic acid (LPA) was assayed at various concentrations in the cell tracking apparatus, and surprisingly showed a dose-dependent induction of locomotion and at the optimum concentration of 20 μ M, a speed of 11.4 ± 0.9 μ m/min, similar to fMLP (11.3 ± 1.0 μ m/min) and IL-8 (10.7 ± 0.6 μ m/min). Polarisation studies indicated a strong correlation between shape change and speed of locomotion, being detectable at 2 μ M LPA and virtually 100% of cells polarised at 20 μ M LPA. At higher concentrations shape changes and motility were diminished, but this decrease was reversed by albumin.

LPA also inhibited the formyl-methionyl-leucyl-phenylalanine (fMLP) and Phorbol 12-myristate 13-acetate (PMA)-induced metabolic burst of human neutrophils assayed using 7-dimethylamino-naphthalene-1,2-dicarboxylic acid hydrazide (DNDH)-dependent chemiluminescence. Inhibition of the PMA-induced metabolic burst by LPA was not affected by pertussis toxin, showing that the effect was not mediated by pertussis toxin-sensitive heterotrimeric G protein, and that inhibition of the PMA-stimulated metabolic burst by LPA could result from a direct action of LPA on the small cytosolic GTP-binding proteins. These results indicate that lysophosphatidic acid production by thrombin-activated platelets could play a significant role in the regulation of the inflammatory response.

INTRODUCTION

1.1.) General Review

Inflammation can be defined through its four classic symptoms 'Dolor, tumor, calor, and rubor', i.e. Pain, swelling, heat and redness. These are beneficial responses, but there is another aspect of long term inflammation, namely, tissue destruction by activated phagocytes.

Most non-steroidal anti-inflammatory strategies aimed at reducing pain do so at the risk of greater or lesser cumulative self-destruction. Most aspects of these four attributes of inflammation are controlled by other cell types, e.g. mast cells and macrophages, but the effectors of tissue damage are primarily the neutrophils. In contrast, administration of glucocorticoid steroids reduces the four attributes of inflammation and diminishes the tissue destruction, but here too there is a high price to pay. Glucocorticoids act globally, affecting the function of many cell types, as well as immune cells, and in doing so, cause a whole range of unwanted side-effects.

The primary aim of much anti-inflammatory therapy research is to discover a second messenger of glucocorticoid action specific to the immune system. This will allow the development of a non-steroidal anti-inflammatory therapy that has the same anti-inflammatory effect as glucocorticoids, but with immune system specificity, and none of the unwanted side-effects.

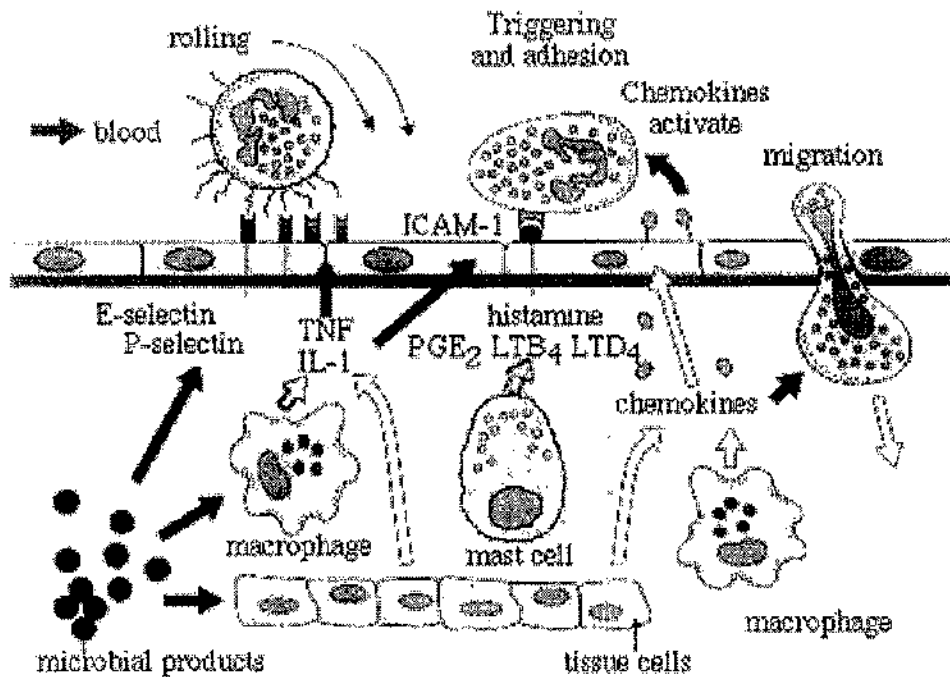
In contrast to the adaptive immune response involving specific antibodies derived from lymphocytes recognising specific foreign antigens, the innate immune response is non-specific response to infectious agents or injury. The cells that mediate the innate response are primarily phagocytic cells, including monocytes, macrophages, and neutrophils, which respond to mediators secreted by other cell types e.g. endothelial,

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mast, or platelet cells, prompting them to leave the circulation (neutrophils and monocytes), and migrate towards the site of insult, where they bind to, engulf, and kill micro-organisms. These phagocytic cells are aided in their role by soluble mediators of immunity, such as, complement and acute-phase proteins, which bind to micro-organisms (opsonization), promoting their uptake by phagocytes.

The recruitment of leucocytes from the blood stream to extra vascular tissue is crucial in host defence against microbial pathogens and in repair of tissue damage. This process involves alteration in the microvasculature of the post-capillary venules, allowing 'leakage' of plasma (Ryan and Majno, 1977) and the movement of leucocytes from the bloodstream to sites of tissue damage, or microbial infection. Studies have indicated that the recruitment of leucocytes from the bloodstream is a multi-step process (Carlos and Harlan, 1994) involving an initial 'rolling' of the leucocyte along the vascular endothelium mediated through a group of adhesion molecules called selectins (Ley et al., 1991). This is followed by stronger adhesion via integrins, a 'switching on' of the leucocytes' motility, diapedesis through the endothelium and migration through extracellular tissue towards a site of inflammation in response to a chemotactic gradient (Carlos and Harlan, 1994) (Fig 1). There are numerous chemical mediators involved in the recruitment of leucocytes from the bloodstream, termed cytokines and chemokines, acting on many cell types including endothelia, epithelia and leucocytes. Most of these mediators are soluble, but some are presented on the surface of the cells. These include both pro- and anti-inflammatory molecules which act together to form an appropriate response to an inflammatory stimulus. Inappropriate inflammatory responses can cause a great deal of tissue damage, as phagocytic leucocytes release neutral proteases capable of destroying tissue at physiological pH and highly active superoxide ions.

Fig. 1. Schematic diagram of leucocyte recruitment to an inflammatory site



Release of pro-inflammatory mediators by tissue, macrophages, and mast cells leads to the activation of the vascular endothelium accompanied by an increase in vascular permeability, and strong adhesion of rolling leucocytes. This is followed by activation of leucocytes, shape change, and locomotion, followed by diapedesis, and chemotaxis to the inflammatory site. The diagram shows some of the pro-inflammatory mediators involved with this process (after Roitt, 1998).

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There are a large number of disease states in humans where these inappropriate immune responses can cause acute or chronic illness, e.g. rheumatoid arthritis, asthma and inflammatory bowel syndrome.

1.1.1.) Chemical mediators of Inflammation

A normal acute inflammatory response involves changes in the microvasculature, the most important change being the flow of plasma exudates and leucocytes from the bloodstream to the damaged tissue.

A number of different groups of molecules involved in signalling in inflammation are produced by immune and non-immune cells. These include, cytokines, which are regulatory, produced by both immune and non-immune cells, signalling a responding cell to change its function; lymphokines, referring to lymphocyte source of cytokine; chemokines, that includes two families of cytokines that induce chemotaxis, the C-X-C and the C-C family; and interleukins, that are mediators, signalling between immune cells. The categorisation of interleukins, however, is not consistent, for some interleukins are not known as such. e.g. Tumour necrosis factor (TNF α), Monocyte chemotactic protein-1 (MCP-1) etc. whereas IL-1, IL-2..... etc. are, despite there not being a difference in function. There are also many lipid mediators of inflammation, e.g. Leukotriene B₄ (LTB₄), which is chemotactic for neutrophils and macrophages, complement components, such as C5a, which is also chemotactic for neutrophils and macrophages, and bacterial products, like the frequently used chemotactic factor, n-formyl-methionyl-leucyl-phenylalanine (fMLP), and lipopolysaccharides (LPS).

The signalling that goes on between immune and non-immune cells via cytokines is often referred to as a 'network'. The intricacies of this

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network however, are far from being fully understood, and only a few pieces of this network have so far been elucidated.

1.1.2.) Chemokines

Chemokines a sub-class of cytokines chemoattractant for immune cells, are secreted by many cell types. They are divided into two families, C-X-C and C-C, that have different cell targets. The C-C and C-X-C refer to the first two cysteines in a conserved motif, and whether they are adjacent or not (Schall, 1991). In general, the members of the C-X-C chemokines are chemoattractant for neutrophils, but not mononuclear leucocytes or other PMN leucocytes (Oppenheim et al., 1991), whilst C-C chemokines are chemoattractant for monocytes, basophils, eosinophils and some T-cell types, but not neutrophils (Schall, 1991).

α -chemokines (C-X-C) include, IL-8 which is important in neutrophil infiltration into inflammatory sites; GRO- α , - β , - γ are chemotactic for, and prime human neutrophils, making them more receptive to further stimulation; melanoma growth-stimulating activity (MGSA), neutrophil activating protein (NAP-2), Platelet Factor 4 (PF4), epithelial-derived neutrophil activating protein (ENA-78), granulocyte chemotactic protein 2 (GCP-2), IP-10, and MGSA. IL-8 was the first of the α -chemokines to be described, and is the one on which most study has been done. It has two membrane-bound receptors (7 span), which couple to G protein domains (Kupper et al., 1992), found in large numbers on neutrophils (20-90000/cell), with fewer present on monocytes and macrophages (1600-8000), and 150-1500 on lymphocytes, so IL-8 is not exclusivity a neutrophil activator, as other cell types are able to respond to it. One of these receptors binds IL-8 specifically, whilst the other binds several

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other α -chemokines. Most α -chemokines share a conserved N-terminal sequence, and contain the tri-peptide sequence Glu-Arg-Leu (ERL) (Clark-Lewis et al., 1991). IL-8 is released by many cell types in response to agents such as IL-1/, TNF α , or Lipopolysaccharides (LPS) (Baggiolini et al., 1989).

β -chemokines (C-C) with a much wider range of target cells, also act through G protein-coupled receptors (Sozzani et al., 1993). β -chemokines include, RANTES, which is chemotactic for monocytes and T lymphocytes (Schall et al., 1990), monocyte chemotactic protein-1 (MCP-1,-2,-3), macrophage inflammatory proteins 1 (MIP-1) and MIP-1.

All of these chemokines are low molecular weight proteins between 7 and 16 kDa, which elicit functional alterations in their target cells, and prime them to respond to other stimuli, as well as inducing release of other inflammatory mediators. The specificity of cell targets may help to explain the specificity of immune cell types found in different organs of the body after an inflammatory insult e.g. eosinophils in the lung in asthma (Butcher, 1991). Increased levels of these chemokines have been associated with a large number of inflammatory diseases, including rheumatoid arthritis, asthma, lung reperfusion injury, and granulomatous disease, so they have been the target of anti-inflammatory therapy research (review; Howard et al., 1996).

1.1.3.) Lipid mediators of Inflammation

A wide range of lipid mediators are involved in the inflammatory process, some of which have anti-inflammatory effects, others pro-inflammatory. The majority of lipid mediators are products of

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arachidonic acid (AA), which is liberated from membrane phospholipids by the action of phospholipase A₂.

Eicosanoids, including prostaglandins, leukotrienes and thromboxanes all have 20 Carbon atoms, and their name contains a number reference to the number of double bonds contained in the molecule. They have diverse effects on various cell types and differing effects on the same cell types in different organs in the body. The variety of effects can be explained in part by the existence of selective receptors for different eicosanoids. Elevated levels of eicosanoids are associated with a number of inflammatory disorders, e.g. in lung tissue is associated with bronchoconstriction in asthma (Wenzel et al., 1990; Liu et al., 1990).

1.1.3.1.) Prostaglandins

Prostaglandins (PGs) are produced from AA by the action of cyclo-oxygenase enzymes, CoxI and CoxII. PGs named PGA through PGI₂, are short-lived, so act as local mediators, but their role in inflammation is not fully understood, as they have been described as having both pro- and anti-inflammatory effects. The expression of Cox I and II and their activation has been linked to the opposing effects that prostaglandins seem to possess, with CoxI being constitutively expressed, and responsible for the protective, anti-inflammatory effects, whilst CoxII, the inducible type being responsible for the pro-inflammatory role.

In their pro-inflammatory role, PGE, PGE₂, PGI₂ cause vasodilation (Williams and Peck, 1977), acting in synergy with C5a, LTB₄, and histamine to produce edema, mediate fever and myalgia in response to IL-1, and in synergy with bradykinin to potentiate pain. They can also inhibit the release of vasoconstriction mediators like endothelin-1, by

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inhibiting transcription of mRNA and secretion of protein (Prins et al., 1994).

As anti-inflammatory mediators they are able to inhibit leucocyte recruitment (Asako et al., 1992). There is also an increase in the symptoms of mucosal inflammation in animal models of colitis when animals are treated with inhibitors of prostaglandin synthesis (Wallace et al., 1992). PGE₂ is an inhibitor of some pro-inflammatory cytokines, namely, TNF α release (Kunkel et al., 1986) and transcription (Kunkel et al., 1988) as well as IL-1 (Kunkel and Chensue, 1985) from macrophages, and also inhibits the release of PAF, histamine and TNF α from cultured mast cells (Hogaboam et al., 1993). They have anti-inflammatory effects on neutrophils, inhibiting release of LTB₄ (Ham et al., 1983), IL-8 (Wertheim et al., 1993), and suppressing superoxide release from these cells (Wong and Freund, 1981).

1.1.3.2.) Leukotrienes

Leukotrienes are produced from AA by the action of the enzyme, 5-lipoxygenase. There are two main types of leukotrienes, LTB₄, and peptido-leukotrienes LTC₄, LTD₄, and LTE₄, that have amino acid residues attached. Leucocytes, endothelial, and epithelial cells all produce the peptido-leukotrienes, whilst the neutrophil is the main source of LTB₄, although it is also produced by macrophages and monocytes. Release of leukotrienes is induced by cytokines, as well as activation through adhesion of leucocytes, this induction being reduced by antibodies to CD18 and L-selectin (Brady and Serhan, 1992).

LTB₄ is a pro-inflammatory mediator, chemotactic for neutrophils, but displays no effect on vascular permeability (Martin et al., 1989). It

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induces increased adhesion of neutrophils to endothelial cells (Hoover et al., 1984; Palmbald and Lerner, 1992) and stimulates release of superoxide ions by neutrophils. Administration of LTB₄ in *in vivo* models of inflammation causes increased adhesion and migration of leukocytes, as well as increased vascular plasma leakage (Thureson et al., 1986). Increased levels of LTB₄ in inflammatory bowel disease (Sharon and Stenson, 1984), and rheumatoid arthritis (Davidson et al., 1983), indicate that it is a mediator in these conditions. It also causes the proliferation of T lymphocytes through increased production of IL-2, and the production of cytokines by monocytes (Rola-Pleszczynski et al., 1986; Rola-Pleszczynski 1992), acting through the transcription factors NF κ B (Brach et al., 1992) and AP-1 (Stankova and Rola-Pleszczynski, 1992). Peptido-leukotrienes display no chemotactic activity but stimulate smooth muscle contraction (Samuelsson et al., 1987), increase vascular permeability (Peck et al., 1981; Joris et al., 1987), can induce increased P-selectin expression on endothelial cells (Kanwar et al., 1995). LTD₄ induces IL-1 expression by monocytes, INF γ secretion by T lymphocytes (Johnson et al., 1986), and increased expression of these lipids has also been implicated in inflammatory bowel disorders (Peskar et al., 1986) and asthma (Wenzel et al., 1990).

1.1.3.3.) Thromboxanes

Thromboxanes also produced via cyclo-oxygenase enzyme pathway, are primarily released by platelets, however, neutrophils also release them (Higgs et al., 1983). Thromboxane is a potent vasoconstrictor and stimulator of platelet aggregation. It can also induce LTB₄ release and so has a role in the recruitment of leucocytes to an inflammatory site.

1.1.3.4.) Lipoxins

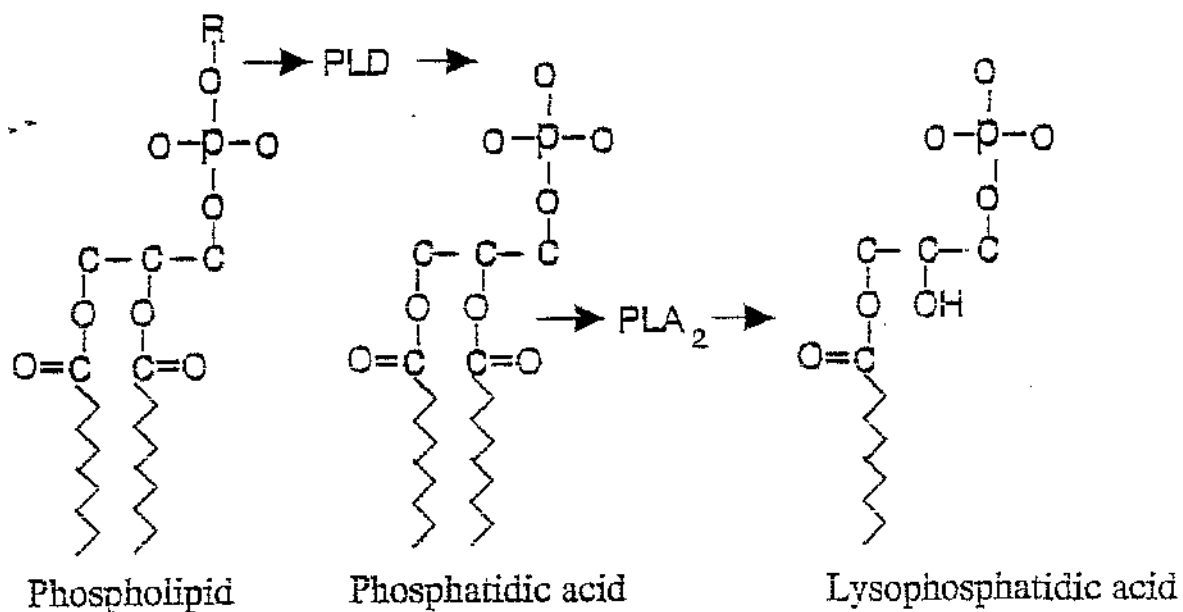
Lipoxins are a group of lipids produced via lipoxygenase enzyme activity on AA, via 15-HETE, which is a product of the aspirin-acetylated CoxII enzyme. Lipoxin A₄ has some anti-inflammatory effects in *in vivo* animal models of inflammation, eliciting vasodilation and increase in blood flow, without plasma exudation or leukocyte adherence (Dahlen et al., 1987), so some of the anti-inflammatory effects of aspirin could be ascribed to the diversion of AA metabolism to the lipoxin pathway via 15-HETE.

1.1.3.5.) Phospholipids

1.1.3.5.1.) Lysophosphatidic Acid

Lysophosphatidic acid (LPA), 1-acyl-*sn*-glycerol 3-phosphate (Fig 2), mainly C16:0 and C18:0 is released from thrombin-activated platelets as a mixture of 1-palmitoyl and 1-stearoyl derivatives (Eichholtz et al., 1993). It can be produced by the action of phospholipase A₂ (PLA₂) on phosphatidic acid (PA), which itself can be produced by phospholipase D action on phosphatidylcholine (PC) or phosphatidylethanolamine (PE) (Fig. 2). Platelets are known to have a PA-specific PLA₂ (Billah et al., 1981). LPA has a variety of biological effects when exogenously applied to various cell types. These include, stimulation of DNA synthesis, cell proliferation (van Corven et al., 1989), calcium mobilisation, platelet aggregation and adenylyl cyclase (Moolenaar, 1991), inhibition of cAMP-induced stellation (Edwards et al., 1993), neurite retraction (Jalink et al., 1993), stimulation of focal adhesion and actin stress fibres through activation of ras-related GTP-binding protein rho (Ridley and Hall,

Fig. 2. Schematic diagram showing the actions of PLA₂ and PLD on phospholipids producing lysophosphatidic acid



R-residue; choline, ethanolamine.

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1992), and activation of Phospholipase C (Moolenaar, 1991; van Corven et al., 1991; Pelvin et al., 1991; Naccache et al., 1991). A 55 kDa putative receptor for LPA has been described (van DerBond et al., 1992) and it appears to be an intrinsic membrane protein (7 span) characteristic of the type that binds trimeric G-proteins. LPA has been reported to affect cell functions through a family of membrane-bound G-proteins, only some of which show sensitivity to pertussis toxin (van Corven et al., 1993). LPA is rapidly released from thrombin-activated platelets and is found in serum bound to serum albumin at biologically active concentrations, and so has been implicated in the proliferation of cells and the control of inflammation in response to injury.

Engleberger et al., 1987, assessed the effect on the metabolic burst of polymorphonuclear leucocytes (PMNs) of a number of lipid species including lysophosphatidylcholine (LC), platelet activating factor (PAF), and its precursor lyso-PAF and found that they enhanced the release of superoxide ions, when stimulated by 4-O-Phorbol-12-myristate-13-acetate (PMA). They postulated that these lipids might be important in the activation of PMNs during tissue injury or in the inflammatory response. However, they did not study the effect of LPA on the metabolic burst of PMNs. No receptor has been found on human neutrophils until now, and it has been shown that there is no change in intracellular calcium levels in neutrophils when LPA was applied extracellularly at concentrations that cause an increase in cultured fibroblasts (Jalink et al., 1990). Since LPA is found at biologically active concentrations at sites of inflammation and tissue injury, it may have an important role in the regeneration of tissue, since it promotes fibroblast cell proliferation (van Corven et al., 1989), and it is possible that it will have an effect on the functions of immune cells at these sites *in vivo*.

1.1.3.5.2.) Platelet Activating Factor

Platelet activating factor (PAF), 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine was originally named through its ability to cause aggregation and degranulation in platelets (Benveniste et al., 1972), however, since then it has been shown to have many other biological functions, acting on, and being released by many cells involved with the inflammatory response. It causes activation of neutrophils through increasing their adhesion to endothelium (Kubes et al., 1990) by increasing expression of β 2-integrins (Sun et al., 1996), and indeed has been shown to act as an adhesion molecule itself through surface expression of PAF on endothelial cells (Zimmerman et al., 1990) and priming them for superoxide release (Engleberger et al., 1987).

1.1.4.) Adhesion molecules and cytokines involved in recruitment of leucocytes to a site of inflammation.

Adhesion between immune cells and cells of the microvasculature plays an important role in the inflammatory response. The first step in immune cell activation is through adhesive interactions. Many adhesion molecules are involved in this process, some of which are constitutively expressed on both immune and endothelial cells, whilst others are expressed only following activation by an inflammatory signal. The expression and activation state of these adhesion molecules is controlled by both pro-inflammatory factors to evoke an inflammatory response, and must be regulated by anti-inflammatory mediators to prevent undesirable inflammation in tissue. This appears to be the most attractive stage to block inflammation and has been the subject of a great deal of

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investigation. The importance of adhesion between leucocytes and the endothelium can be demonstrated in diseases such as leucocyte adhesion deficiencies (LAD), where the patients suffer persistent and life-threatening infections (Anderson and Springer, 1987).

There are a large number of adhesive interactions between immune and non-immune cells during inflammation, involving a number of different groups of adhesion molecules and ligands.

1.1.4.1.) Selectins

The first stage in the recruitment of leucocytes to an inflammatory site involves weak interactions between the endothelium and the leucocyte via a group of cell adhesion molecules called selectins (Carlos and Hanlan, 1994). There are three main family members involved in this process. They are named after the cells on which they were first identified; L- from Leucocytes, P- from Platelets, and E- from Endothelial cells, but are also expressed on other cell types. At any time in the body a large number of leucocytes are interacting with the vascular endothelium via selectins. These cells are referred to as the marginating pool. As the name suggests each of the three selectin family members contains a lectin domain, that is responsible for their interaction with ligand. The ligands for selectins were shown by inhibition studies to be carbohydrate in nature (Stoolman and Rosen, 1983). Further studies have indicated that particular moieties found at the termini of oligosaccharide chains were important to the binding of selectins, including sialylates and fucosylates, the most prominent of these being the Sialyl Lewis X moiety (Walz et al, 1990; Zhou et al., 1991). In patients with leucocyte adhesion deficiency type II (LAD II), where there is a deficiency in fucose metabolism, there

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is a reduction in the migration of neutrophils into tissue (Price et al., 1994), with a susceptibility to repeated infection, although not as severe as that for LAD I defect; section 1.1.4.2. Studies have shown that LAD II neutrophils were only able to adhere to vascular endothelial cells under low flow conditions, whereas LAD-1 neutrophils were able to roll on the endothelial cells, but were unable to adhere strongly (von Adrian et al., 1993). These findings support the rolling, adhesion followed by migration model of leucocyte migration from the bloodstream. Calcium is also important for ligand binding as shown by inhibition of adhesion by EGTA (Bevilacqua and Nelson, 1993).

1.1.4.1.1.) E-selectin

E-selectin (CD62E, ELAM-1) is a 110 kDa selectin adhesion molecule, expressed only on endothelial cells where it is synthesised following stimulation by cytokines $\text{TNF}\alpha$, IL-1 or LPS (Bevilacqua et al., 1987, 1989). It is stimulated at the level of transcription, as inhibitors of transcription and translation prevent its expression following stimulus (Bevilacqua et al., 1989). Expression of E-selectin on a cultured endothelial monolayer is transient peaking at 3 to 6 hours following stimulation, and decreasing to basal levels after about 24 hours even in the continued presence of the stimulus. Induction of E-selectin expression, like many other genes involved with the inflammatory response, is via $\text{NF}\kappa\text{B}$ (Collins et al., 1995). Other factors may influence the expression of E-selectin. Treatment of the endothelium with interferon-gamma ($\text{IFN}\gamma$), and either $\text{TNF}\alpha$ or LPS, prolongs the expression of E-selectin. Treatment with glucocorticoids decreases the thrombin and LPS induction

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of E-selectin expression (Cronstein et al., 1992), and may explain some of the anti-inflammatory actions of glucocorticoids.

1.1.4.1.2.) L-selectin

L-selectin (CD62L, Lam-1, LECAM-1) is a 70-90 kDa transmembrane protein. It is constitutively expressed on the surface of leucocytes (Juttila et al., 1989) and differs from E- and P-selectin in that it is down-regulated upon activation of the leucocyte by cytokines like IL-8, through shedding of the extracellular component of the molecule (Kishimoto et al., 1989). It is expressed on all circulating leucocytes except a sub-population of lymphocytes.

The interaction of leucocytes with the endothelium via selectins is normally referred to as rolling, since the leucocytes maintain movement, at a reduced velocity. L-selectin, as well as P-selectin, are required for leucocyte rolling as shown in *in vivo* studies using antibodies to the two molecules (Ley et al., 1991; Juttila et al., 1989; Bosse and Vestweber, 1994).

1.1.4.1.3.) P-selectin

P-selectin is a 110 kDa selectin adhesion molecule. The gene for P-selectin is constitutively expressed in platelets and endothelial cells, however, the protein is stored within alpha granules in platelets and Weibel-Palade bodies in endothelial cells (McEver et al., 1989). The protein is only expressed on the surface of the cell following exposure to an appropriate pro-inflammatory agent such as leukotriene C₄/D₄

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(Kanwar et al., 1995), histamine, thrombin, or oxygen radicals, which cause fusion of the granules with the plasma membrane, with maximum expression seen after only 10 minutes (Hattori et al, 1989). This surface expression is transient however, and the protein is internalised rapidly (McEver et al., 1989), or shed like L-selectin. $\text{TNF}\alpha$, LPS or IL-1 induce increased P-selectin gene expression without the rapid surface expression, with peak expression 2 to 4 hours later (Gotsch et al., 1994). These two levels of control of P-selectin functional expression on the surface of endothelium leads to fine control possibilities over leucocyte adhesion, which has been shown to be at least partly involved with the initial rolling interaction between leucocytes and the endothelium during an inflammatory response (Bosse and Vestweber, 1994). Upregulation of P-selectin is concurrent with an upregulation and secretion of PAF, which induces CD11/CD18 expression in leucocytes (Zimmerman et al., 1990).

1.1.4.2.) β_2 -integrins

Activation of vascular endothelium by pro-inflammatory mediators, such as $\text{TNF}\alpha$, IL-1 or LPS emanating from an inflammatory site, causes an increase in surface expression of endothelial adhesion molecules (Pober et al., 1986) and the release of further pro-inflammatory both soluble and presented on the endothelial cell surface, such as IL-8 (Rot, 1992) cause the activation of the 'rolling' leucocyte and their adhesion is increased by the activation of a group of adhesion molecules called β_2 integrins (Lo et al., 1991; Diamond and Springer, 1994). This is followed by an activation of cell motility and shape change (Carlos and Hanlan, 1994).

There are two main β_2 -integrins responsible for the strong adhesion of activated neutrophils to vascular endothelium, namely, LFA-1,

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(CD11a/CD18) (Marlin and Springer, 1987) and Mac-1 (CD11b/CD18) (Diamond et al., 1990).

LFA-1 is expressed on hematopoietic cells, lymphocytes, monocytes, macrophages, neutrophils and eosinophils. It is a heterodimer, made up of the 180 kDa CD11a subunit non-covalently associated with the 95 kDa CD18 subunit. Mac-1 is expressed on monocytes, macrophages, natural killer (NK) cells and neutrophils. It is a heterodimer, made up of the 170 kDa CD11b subunit and is also non-covalently associated with the 95 kDa CD18 subunit.

Their primary ligand is the endothelial intercellular adhesion molecule-1 (ICAM-1) (Diamond et al., 1991). CD11a/CD18 and CD11b/CD18 were shown using antibodies *in vitro*, each to contribute 50% of adhesion of neutrophils to inactivated endothelium (Lo et al., 1989; Smith et al., 1989). CD11a/CD18, however, was shown to be responsible for almost all adhesion to activated endothelium. Antibodies against ICAM-1 reduce neutrophil adhesion by 50% in both cases (Smith et al., 1988), implying that 50% of CD11a/CD18 and CD11b/CD18 mediated adhesion is through other ligands than ICAM-1.

In unstimulated cells these molecules are in an inactive form, and require stimulation to increase their avidity of binding to ligand. Control of this activation is thought also to be crucial in controlling the timing and duration of an inflammatory response, as the strong adhesion of activated neutrophils to the vascular endothelium is a very important step in the migration of the cells to an inflammatory site. This can be shown most clearly in disease states where function of these adhesion molecules is deficient. Leucocyte adhesion deficiency (LAD-1) is a disease associated with the absence of $\beta 2$ integrin function. This disease causes recurrent and potentially life-threatening bacterial infections in sufferers (Anderson and Springer, 1987). There is, however, a CD18-independent pathway to

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neutrophil adhesion and migration into tissue, as seen in post-mortem examinations of LAD-1 patients where neutrophils are found in lung alveolar spaces (Hawkins et al., 1992).

1.1.4.3.) Intercellular adhesion molecules

Intercellular adhesion molecule-1 (ICAM-1) is a 90 kDa cell surface Glycoprotein, with five Immunoglobulin-like domains, that is constitutively expressed on a number of cell types including epithelial (Wegner et al., 1990) and endothelial cells, dendritic cells, monocytes, B cells, T cells, and fibroblasts. Endothelial cell expression of ICAM-1 is increased after stimulation with cytokines such as $\text{TNF}\alpha$, IL-1, IL-8 or Lipopolysaccharide (LPS), over a 24-hour period. This induction causes *de novo* synthesis of ICAM-1 through an increase in gene expression. There is also a much more rapid response in the induction of ICAM-1 following stimulation by thrombin and hydrogen peroxide, assayed by an increase in neutrophil adhesion, which is thought to occur through an alteration in the function of constitutively expressed ICAM-1, as no protein synthesis is required. ICAM-1 is responsible for strong leucocyte adhesion to endothelium during an inflammatory response (Rothlein et al., 1986), and is the main ligand for the β_2 -integrins CD11a/CD18 and CD11b/CD18 (Diamond et al., 1991), which are expressed on all leucocytes, although they have been shown to interact with two other ICAM family members; ICAM-2 and ICAM-3. The expression of ICAM-1 is reduced by glucocorticoid treatment in *in vitro* studies (Cronstein et al., 1992).

ICAM-2 is a 55 kDa integral membrane protein with two immunoglobulin-like domains that are similar to the two N-terminal

domains of ICAM-1, but only binds CD11a/CD18 and not CD11b/CD18 (Staunton et al., 1989). It is expressed on endothelial cells, lymphocytes, monocytes, NK cells and platelets. ICAM-2 is likely to be responsible for ICAM-1 independent leucocyte adhesion, as shown by studies using monoclonal antibodies to CD11/CD18 but not ICAM-1, completely inhibited the homotypic aggregation of lymphocytes (Rothlein et al., 1986).

ICAM-3 has similarity of structure to ICAM-1, is not expressed on vascular endothelium, but is constitutively expressed on inactivated leucocytes (Fawcett et al., 1992).

1.1.4.4.) Platelet-endothelial cell adhesion molecule

Platelet-endothelial cell adhesion molecule (PECAM-1) (EndoCAM, CD31) is a cell adhesion molecule first discovered on platelets (Metzelaar et al., 1991) and endothelial cells (Albelda et al., 1990), but has since been shown to be expressed on many other cell types, including monocytes, neutrophils, NK cells, and about 50% of all lymphocytes (Watt et al., 1995). It is involved in homotypic adhesion of endothelial cells (Albelda et al., 1991), and the adhesion of platelets to myeloid cells (Muller et al., 1992). Binding of PECAM-1 to ligand has also been implicated in activation of leucocytes, as antibody binding to PECAM-1 induced the respiratory burst in monocytes, but notably, not in neutrophils (Stockinger et al., 1990).

Antibody studies both *in vitro* (Muller et al., 1993), and *in vivo* (Vaporciyan et al., 1993) have shown that it is required for leucocyte transmigration across the endothelium. β_2 -integrins have also been implicated as a requirement for transmigration. A hypothesis is that

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ICAM-1 molecules are distributed in such a way on endothelial cells that they guide the leucocytes towards the junctions between endothelial cells, where the majority of PECAM-1 is expressed (Muller et al., 1989), and leucocyte PECAM-1 interactions are responsible for the movement of leucocytes through the endothelium.

1.1.4.5.) Vascular cell adhesion molecule-1 (VCAM-1)

Vascular cell adhesion molecule-1 (VCAM-1, CD106) is a 110 kDa transmembrane adhesion molecule, found on endothelium only after cytokine activation. Its ligand is primarily the β_1 -integrin, VLA-4, expressed on most circulating leucocytes. Antibody studies have indicated that it has a role in the transendothelial migration of monocytes and eosinophils (Hakkert et al., 1991), and is also responsible for interactions between endothelium and lymphocytes (Rice et al., 1990; Oppenheim et al., 1991). Although there is a marked reduction in the infiltration of neutrophils into tissue in leucocyte adhesion deficiency diseases where there is dysfunction of CD18 adhesion molecules, there is infiltration of eosinophils, lymphocytes, and some monocytes (Anderson et al., 1985). VCAM-1 has been implicated as the CD18-independent pathway of leucocyte adhesion and transendothelial migration. The lack of expression on some cell types and selective expression of ligands for such adhesion molecules, may explain the selective targeting of cell types in the immune system into particular tissues.

1.1.5.) Anti-inflammatory mediators

There are a number of proposed anti-inflammatory mediators that act in conjunction with the pro-inflammatory mediators to control the extent of, and 'switch off', the inflammatory process. The definition of a molecule as being either pro- or anti-inflammatory can be a difficult exercise as many factors display characteristics of both, depending on the assay conditions, levels of expression, cells studied, and parameters being measured.

1.1.5.1.) Lipocortin-1

Lipocortin-1 (LC-1), a 37kDa (343 amino acid) protein is a member of the annexin family of proteins that show calcium and phospholipid binding properties. Each member of the Annexin family contains a core of 4 or 8 repeated 70 amino acid domains (Crompton et al., 1988), and within each is a 17 amino acid consensus sequence motif, which is involved with calcium and phospholipid binding (Geisow et al., 1986). LC-1 has a unique N-terminus sequence conferring specific structural and functional properties. It was first identified in 1979 in Guinea-pig model of inflammation, and was described as a potential mediator of the anti-inflammatory effects of glucocorticoids (Flower and Blackwell, 1979). The Lipocortin-1 gene was first cloned in 1986 (Wallner et al., 1986), and contains 13 exons, the first being non-coding. The promoter region contains TATA and CCAAT sequence as well as having a glucocorticoid response element (GRE) in the region. Gene expression has been shown to be restricted to specific cell types such as, ductal/airway epithelia, macrophages and polymorphonuclear leucocytes (Fava et al., 1989) and is

inducible by glucocorticoids. The anti-inflammatory role of LC-1 has been debated for a number of years, with contradictory evidence being presented by various groups. The anti-inflammatory role of LC-1 is described further in section 1.1.9.

1.1.5.2.) Anti-inflammatory peptides

There are a growing number of peptides that have apparent anti-inflammatory effects in *in vivo* models of inflammation (review, Wei and Thomas, 1993). These include Corticotropin-releasing factors (CRFs) and related peptides that are able to inhibit edema formation in animal models of inflammation. These peptides are so called for their ability to affect the synthesis and release of corticosteroids from the adrenal glands. However, the effect they have on edema is not through the release of corticosteroids, as shown by their ability to reduce edema in adrenalectomized or hypophysectomized rats, implying a more localised effect on vascular permeability. The di-peptide sequence K-R (Lys-Arg) appear to be important for activity of these peptides.

Neurotensin and similar peptides (NTs) e.g. Xenopsin, that have the Lys-Arg sequence present are also able to inhibit edema formation in animal models of inflammation. NT was first discovered and described as a pro-inflammatory mediator, increasing vascular permeability, hypotension and cyanosis in the rat. However, local administration causes an inhibition of histamine release. The apparent anti- and pro-inflammatory roles of these molecules depends primarily on their mode of administration.

Both these sets of peptides, CRFs and NTs induce transient hypotension, but this does not appear to be the cause of their anti-inflammatory effects

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as sodium nitroprusside administration also induces this hypotension without affecting edema formation.

Other anti-inflammatory peptides include, vasoactive intestinal peptide (VIP) that inhibits bronchoconstriction and vasoconstriction induced by pro-inflammatory mediators, as well as reducing lung injury induced by hydrochloric acid, oxidants and PAF, and reduction of edema in lung assays; and antiinflammins 1 and 2 which are referred to in section 1.1.9, as they are fragments of Lipocortin-1, thought to have anti-inflammatory properties similar to the native protein.

1.1.5.3.) Adenosine

Adenosine has two receptors, A₁ and A₂ (van Calker et al., 1979), both of which are found on the majority of cells in the body. They effect cell functions through a pertussis toxin sensitive membrane-bound G-protein receptor (Dolphin and Prestwich, 1985). Binding of receptor increases levels of intracellular cAMP, and inhibits cells responding to other stimuli (van Calker et al., 1979; Londos et al., 1980). The anti-inflammatory role of adenosine is not fully understood, but several *in vitro* and *in vivo* studies have implicated it as a potent anti-inflammatory mediator (review; Cronstein, 1994). It inhibits superoxide generation by activated leucocytes and reduces adhesion of leucocytes to activated endothelial cells *in vitro*, and accumulation of leucocytes in inflammatory models *in vivo*.

1.1.5.4.) Nitric Oxide

The role of Nitric oxide (NO) as a cellular signalling molecule first came to light in a study of blood vessel relaxation, and indeed is known in that system as endothelium-derived relaxing factor (EDRF). As the name suggests, it is synthesised by endothelium, and causes relaxation of blood vessels allowing increased blood flow. However, NO has been found to influence the function of virtually every organ and cell in the human body, and indeed, every cell in humans appears capable of producing NO given the correct stimulation (review, Harald et al., 1994). It has been implicated in many cellular systems including, as a neurotransmitter, hormonal functions in the heart, lungs kidney, pancreas gut and the immune system.

Within the immune system NO appears to have a 'split personality', it has apparent pro- and anti-inflammatory functions, depending on the cells being effected and the timing of this effect.

There are three separate gene products producing nitric oxide synthetases (NOS), all of which are calmodulin dependent, but only one of which is independent of increases in intracellular calcium, referred to as inducible nitric oxide synthetase (iNOS), and is induced by agonists such as cytokines and bacterial lipopolysaccharides. Induction of iNOS confers on the cell the ability to produce NO over extended periods. This enzyme is primarily implicated in the immune functions of NO. It confers anti-microbial activity on the host cells, but the cost of this is potential damage to self by NO derived reactive molecules, such as peroxynitrite, produced by the reaction of NO with superoxide ions (Szabo, 1996).

NO inhibits platelet aggregation and adhesion of platelets by raising levels of platelet cGMP in synergy with PGI₂ (Radomski et al., 1987), and also inhibits histamine release by mast cells (Salvemini et al., 1991)

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1.1.6.) Cells of Inflammation

There are a large number of cell types involved in an inflammatory response. Interactions between these cell types via direct interaction, or through soluble mediators are extensive, and mediated by a whole range of both pro- and anti-inflammatory signals. Figure 3 shows the development of the hematopoietic cells primarily involved in the innate immune response.

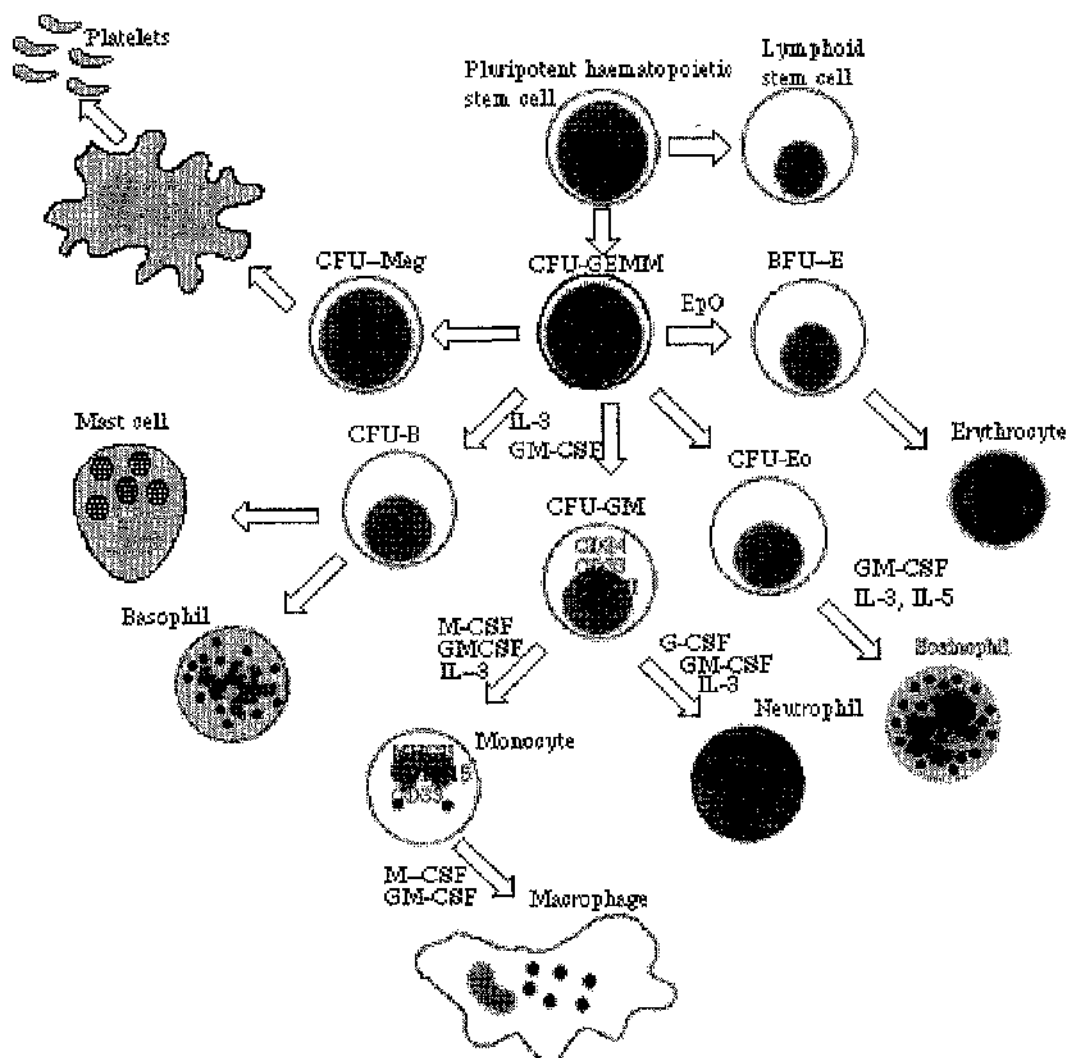
1.1.6.1.) Polymorphonuclear Leucocytes

The most numerous of all immune cells in the circulation are the Polymorphonuclear leucocytes (PMNs), which are made up of three cell types, namely neutrophils, basophils, and eosinophils.

1.1.6.1.1.) Neutrophils

Neutrophils make up more than 90% of PMNs and are the first cells detected at a site of inflammation/infection, where they are thought to be very efficient 'killers' of a wide variety of microbial pathogens. The importance of these cells as the 'first-line' of defence against infection can be seen in individuals where there is neutrophil dysfunction. These individuals suffer repeated bacterial and fungal infections, particularly in skin and mucous membrane, areas of the body freely accessible to microbial infection. Neutrophil dysfunction has two main causes; either a lack of the cells in the blood circulation due to arrest of development/maturation; neutropenia, or a dysfunction preventing the

Fig 3. Haematopoiesis. Differentiation of granulocytes, platelets, mast cells, and erythrocytes(After Roitt).



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cells getting from the circulation into an infected site, or unable to deal effectively with the microbes when they do, due to a defect in cell function. The outcome of these two dysfunctions is similar, in that the individual has recurrent infections; however, they differ in the complications arising from initial infection. In individuals who have neutropenia, the initial localised infection often develops into septicaemia, as the infectious organism is able to escape from the soft-tissue into the blood stream; this is not so prevalent in the latter group (Howard et al., 1977; Anderson et al., 1985).

1.1.6.1.2.) Eosinophils

Eosinophils have been associated with allergic asthma, and are found in relatively large numbers in the lungs of sufferers. Eosinophils, like other leucocytes must adhere to vascular endothelial cells before infiltration into tissue. Unlike neutrophils they express the membrane adhesion molecule VLA-4, which may provide a mechanism for the specificity of their migration into tissue (Butcher, 1991); section 1.1.4.5.

1.1.6.1.3.) Basophils

Basophils found in very small numbers in the bloodstream, are functionally similar to mast cells. They are primed by exposure to IL-3 or IL-5 to respond to stimuli, such as, GM-CSF, MCP-1 or IL-8, releasing granules containing primarily histamine and leukotrienes by exocytosis (Baggiolini and Dahinden, 1994; Dahinden et al., 1989).

1.1.6.2.) Mast cells

Mast cells are important mediators of the inflammatory response, as many of them are found proximal to the vascular-endothelium in all tissues, and they are capable of secreting a large number of pro-inflammatory mediators that will cause the activation of the endothelium and so the recruitment of blood leucocytes to a site of inflammation. They are also sensitive to many of the stimuli found at a site of infection or injury, namely IgE, complement component C5a, neuropeptides, superoxide ions, several lipid mediators, including PAF, LTB₄ and PGD₂, bacterial toxins, histamine and bradykinin. They are also sensitive to physical stress. The presence of mast cells near to the vascular endothelium, makes them suited to amplify the initial inflammatory signal, be it an infectious agent, or a physical injury, by activating the endothelium, increasing vascular permeability, and attracting blood leukocyte invasion of the inflammatory site (review, Kubes and Granger, 1996)

1.1.6.3.) Monocytes and Macrophages

Monocytes and Macrophages were first identified by Metchnikoff, 1893. The bone marrow progenitors of these cells develop over a period of 6 days into blood monocytes. Monocytes have a half-life of approximately 3 days. Some of them migrate into tissue and develop into tissue macrophage, where their life-span can be from days to many months. The mononuclear phagocyte system plays an important regulatory role in inflammation, borne out by the fact that they are capable of secreting around one hundred different products (Nathan, 1987). They interact

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with all other immune cells, modulating their responses in two main ways. Firstly, they process and present antigen to Lymphocytes, which recognise these antigens when bound to Major Histocompatibility complex molecules on the cell surface. Secondly, and more importantly for the purpose of this study, they secrete many important immunoregulatory molecules both pro- and anti-inflammatory, that alter the behaviour of other immune cells. Glucocorticoid administration inhibits monocyte development *in vivo*.(Shezen et al., 1985).

1.1.6.4.) Endothelial cells

For many years the vascular endothelium was seen as nothing more than a barrier, preventing blood cells and plasma proteins from entering body tissues. More recently the role of the endothelium in the control of inflammation has been realised. Endothelial cells are capable of secreting many pro- and anti-inflammatory mediators, can activate leucocyte adherence by alteration of surface expression of adhesion molecules, and alter the extent of homotypic adhesion to increase/decrease vascular permeability. They are also able to present leucocytes with pro-inflammatory molecules bound on their surface, e.g. PAF, which activates CD11a&b/CD18 (Zimmerman et al., 1990). They are involved in the first stage of the activation and movement of leucocytes from the bloodstream to a site of inflammation, so are an obvious target for anti-inflammatory therapy, and indeed some of the actions of anti-inflammatory steroids affect endothelial cell functions. Glucocorticoid administration causes a decrease in vascular permeability, and a decrease in the expression of adhesion molecules ELAM-1 and ICAM-1 on endothelial cells (Cronstein et al., 1992). Preventing the initial interaction

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of leucocytes with the endothelium would appear to be the most promising approach to therapy for inflammatory diseases.

1.1.6.5.) Platelets

Platelets are another cell type whose role in inflammation was overlooked for many years. Their role in blood clotting is well recognised, but the arsenal of inflammatory mediators that they are able to secrete has only recently been realised. They secrete LPA in response to thrombin (1.1.3.5.1), and constitutively express the P-selectin gene and store the gene product in alpha granules, only transporting it to the surface upon stimulation (1.1.4.1.3.). An important role in the inflammatory response by platelets is to aggregate and bind endothelial cells at a site of vascular damage. They then secrete thromboxanes, serotonin and fibrinogen, which act to increase vascular permeability, and activate complement, which in turn induces the recruitment of leucocytes to the site via the chemoattractant complement factor C5a.

1.1.7.) The release of active oxygen species by human neutrophils

The release of superoxide ions by leucocytes is a major part of the arsenal of anti-microbial weapons that these cells possess. However, much of the self-tissue damage caused by granulocytes in chronic inflammatory disorders is thought to be due to the release of these highly reactive and destructive oxygen metabolites. However, on the other hand, if there is a defect in the production of these oxygen species, as in a group of diseases

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known as, chronic granulomatous disease in childhood, the outcome is frequent microbial infections (Tauber et al., 1983).

In recent years many pro-inflammatory mediators have been reported either to activate neutrophils directly or else prime their responses to known stimulators such as fMLP and PMA. The activation of neutrophils by fMLP and PMA is determined by two interrelated cellular signalling pathways; fMLP acts by binding to specific plasma membrane receptors, that are coupled to pertussis toxin-sensitive G proteins and which in turn activate phospholipase C. Activation of this enzyme elevates cytosolic free calcium by means of inositol 1,4,5-triphosphate pathway and also activates protein kinase C (PKC) via the diacylglycerol pathway that phosphorylates several key components of the oxidase system (Thomas et al., 1990) leading to the assembly of the NADPH oxidase complex, which is made up of several protein subunits, and release of superoxide ions. PMA acts as an analogue of diacylglycerol by directly binding to the calcium/phospholipid dependent PKC (Tauber, 1987; Garland, 1992)

1.1.8.) Inappropriate acute and chronic inflammation: Treatment with anti-inflammatory mediators.

It is apparent from the above text that the reaction of the immune system to injury or infection is an intricate and highly controlled reaction involving the interaction of many cell types with one another, via the expression and secretion of a large number of soluble mediators as well as by direct contact through adhesion molecules. The body is continually under threat from fungal and bacterial infection, and so the first line of defence, namely the phagocytic leucocytes, have to be constantly on duty. It is not surprising, when the number of cells involved and the sensitivity

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of the cellular mediated immune system is considered, that sometimes the system of control breaks down, leading to an inappropriate or exaggerated response and resulting self-damage by overactive phagocytic cells secreting neutral proteases, and reactive oxygen species, in diseases such as, rheumatoid arthritis, where joints become inflamed without any apparent insult or injury. The system too, can become oversensitive to stimuli, in diseases such as asthma, where an allergic reaction results from hypersensitivity to an allergen, causing inflammation of lung tissue. The cause of these types of diseases are not known, but many types of drug have been developed to reduce the symptoms. The most widely used anti-inflammatory treatment is administration of glucocorticoid type steroids, which are highly effective in reducing the inflammation by reducing the number of immune cells at the site of inflammation, and therefore reducing tissue-damage. This benefit has to be weighed against the side-effects of this therapy. Non-steroidal anti-inflammatory drugs, tend to alleviate only the obvious symptoms of the inflammation, namely swelling and pain, but do not reduce the number of inflammatory cells at the inflammatory site, so do not prevent the tissue damage caused by these cells, and in the long term the consequences of this inflammation become worse.

1.1.8.1.) Glucocorticoids

Glucocorticoids, and four other major classes of steroid hormones, progestagens, mineralocorticoid, androgens, and estrogens, are synthesised from a common precursor molecule; cholesterol. Glucocorticoids, synthesised in the adrenal cortex, promote the formation of glycogen and enhance the degradation of fat and protein *in vivo*, as

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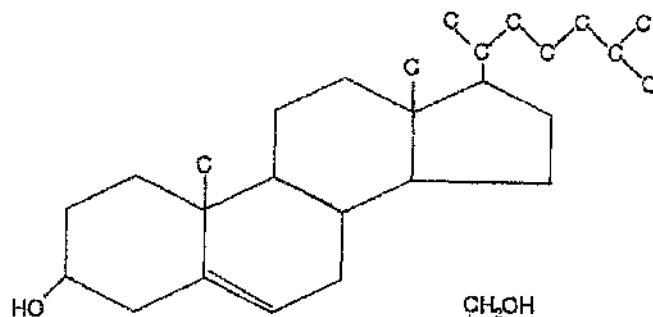
well as their anti-inflammatory functions. Figure 4 shows a diagram of their pre-cursor molecule; cholesterol, and some glucocorticoids commonly used as anti-inflammatory therapeutic agents. As discussed below, they also have a major effect on the development and function of cells involved in the inflammatory response and most of their effects are in down-regulating the inflammatory response, through effects on the development and distribution of leucocytes, inhibiting the release of pro-inflammatory mediators, and reduction of adhesion through down-regulation of adhesion molecules on vascular endothelial cells. Some cytokines, for example IL-6 induce the release of glucocorticoids from the adrenal cortex, as a way of balancing the pro- and anti-inflammatory mediators to promote a measured inflammatory response.

1.1.8.1.1.) Glucocorticoids as anti-inflammatory therapeutic agents

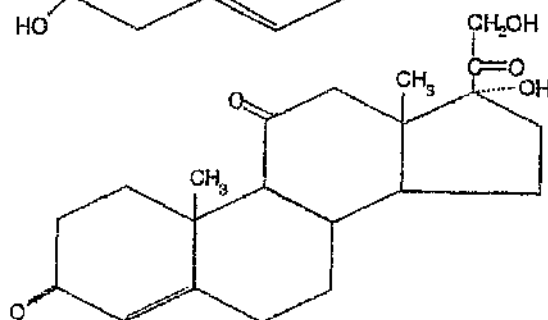
Glucocorticoids were first used as an anti-inflammatory treatment in 1948 , when cortisone was observed to have a very powerful therapeutic effect on patients suffering from arthritis (Hench et al., 1949). However it was soon discovered that there were unwanted side effects from glucocorticoids. This setback however, has not prevented the use of anti-inflammatory steroids for the treatment of many debilitating inflammatory disorders, such as rheumatoid arthritis or osteoarthritis. These disabling conditions mean that the adverse side-effects of steroid administration have to be weighed against the improvement to the quality of life of the patient. "To move freely without restriction due to pain, disability, or weakness is among the most basic of human rights. Any disorder that impairs mobility; limits the capacity to touch, embrace or

Fig. 4. Anti-inflammatory glucocorticoids and their common precursor; cholesterol

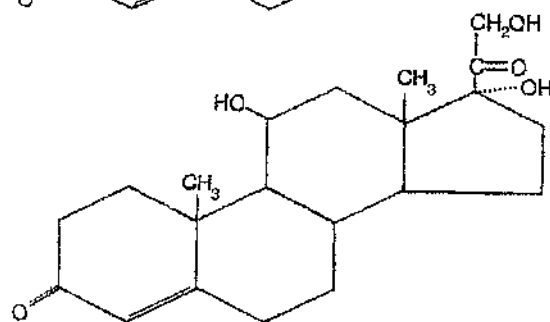
Glucocorticoids, synthesised in the adrenal cortex exert their influences globally, and as well as their anti-inflammatory effects, alter glucose metabolism, enhancing glycogen production, and enhance the degradation fat and protein.



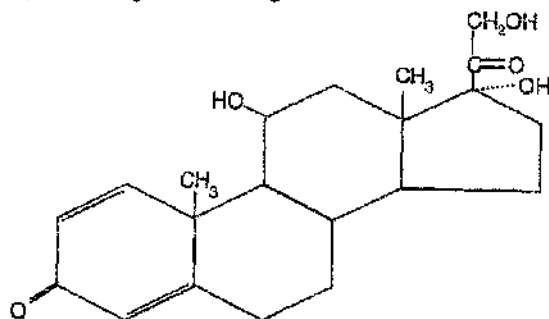
Cholesterol



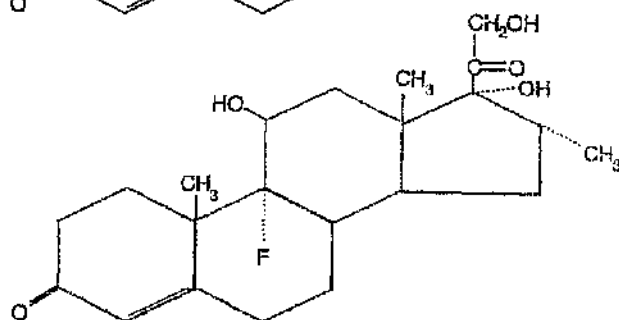
Cortisone



Hydrocortisone



Prednisolone



Dexamethasone

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protect; and interferes with personal hygiene, physical labor, or recreation threatens a person's sense of dignity and self worth" (Sobel and Klein, 1989).

1.1.8.1.2.) Side effects of glucocorticoid administration

Glucocorticoid receptors are found in most cells of the body (Norman and Litwack, 1987), so it is hardly unexpected that there should be side-effects from glucocorticoid treatment, and indeed since they have been used as an anti-inflammatory drug adverse side-effects have been described affecting most organs of the body. Cardiovascular: treatment of preterm infants with pulmonary dysplasia with glucocorticoids has led to hypertension (Fauser et al., 1993) and hypertrophic obstructive cardiomyopathy (Brand et al., 1993); Psychiatric syndromes: Although not common cases have been described of steroid-induced psychotic episodes (Klein, 1992; Mullen and Romans-Clarkson, 1993) Neurological syndromes: including encephalopathy (Durmus et al., 1991), Endocrine and metabolic: adrenal suppression has been described frequently, even with inhaled forms, and may continue after withdrawal of treatment (Zwaan et al., 1992); eyes: ocular hypertension and cataract development (Kaye et al., 1993); Bones and joints: Reduction in bone density (Garton and Reid, 1993), although this appears to be reversed after steroid therapy is withdrawn (Laan et al., 1993); muscle and tendons: reducing the efficiency of respiratory muscle function (Weiner et al., 1993); growth: there has been shown to be a reduction in the rate of growth of children on steroid therapy (Volovitz et al., 1993); and immune defence and infection: Because glucocorticoids, used as anti-inflammatory therapy, suppress the immune system, it follows that the patient under

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steroid therapy will be more prone to infection. Adverse side effects have limited steroids use as anti-inflammatory therapeutic agents, however, a great deal of study is going on to determine the ideal strength and time of dosage, to reduce these side-effects, whilst gaining maximum efficacy of treatment.

1.1.8.1.3.) Molecular mechanism of the anti-inflammatory actions of glucocorticoids

The glucocorticoid receptor (GR) is almost ubiquitous in the cells of the body. The GR is a protein is made up of three domains, the steroid binding domain, the DNA binding domain and the modulatory domain (Meisfield, 1989, 1990; Gieguere et al., 1986). In the absence of ligand, the receptor is bound to a heat-shock protein 90 (HSP90) in the cytosol (Howard and Distelhorst, 1988; Rexin et al., 1988). When steroid binds the GR, the receptor dissociates from the HSP90 protein and translocates to the cell nucleus, where it effects gene expression by binding, as a dimer, to a regulatory sequence known as the Glucocorticoid Receptor Element (GRE) (Scheidereit et al, 1983), which have the consensus sequence d(TGTTCT) (Scheidereit et al, 1986). The binding of receptor to the GRE usually produces a suppression of gene expression (Cairns et al, 1993) of some pro-inflammatory cytokines (section 1.1.8.1.4.), and increased expression of anti-inflammatory mediators like Lipocortin-1 (Flower and Blackwell, 1979). There may be several GREs for a single gene, and these may have differing binding strengths for the receptor dependent on their sequence (Miesfeld, 1989), providing different levels of control of gene expression. A model has been proposed, that genes on which there are more GREs with stronger binding are quickest to be

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affected by glucocorticoid treatment, and the later affected genes have less numerous and/or lower binding affinity GREs (Chalepakakis et al., 1988). The interaction of GR with transcription factors and the alteration of chromatin structure may also have an influence on GR control of gene expression (Adler et al., 1992; Hayes and Wolffe, 1992; Pearce and Yamamoto, 1993; Ptashne, 1988).

There are two subtypes of cellular receptors which bind glucocorticoids: Type I receptors, known as mineralocorticoid receptors, and type II receptors, known as glucocorticoid receptors. Type I receptors have a higher affinity for naturally occurring adrenal steroids, such as cortisol, than type II receptors, and are therefore occupied and activated at lower concentrations of endogenous hormone (Reul and Kloet, 1985; Spencer et al, 1990).

Many of the cytokines, whose expression is inhibited by glucocorticoids do not have a GRE in their promoter region and therefore the mode of repression must be through another mechanism. The transcription factor NFkB, an important transcription factor of cytokine gene expression, is functionally inhibited by glucocorticoids by direct interaction of the GR with subunits of NFkB (Scheinman et al., 1995). This transcription factor is localised in the cytoplasm bound to an inhibitor IkB, which prevents its translocation to the nucleus. Activation of the cell by e.g. cytokines, LPS, reactive oxygen species, or viral infection causes rapid dissociation, phosphorylation and degradation of IkB, and translocation of NFkB to the nucleus (Beg et al., 1993), where it associates with other transcription factors e.g. AP-1, SP1 and C/EBPb to initiate transcription of many different gene products.

1.1.8.1.4.) Anti-inflammatory mechanism of glucocorticoids

The primary anti-inflammatory action of glucocorticoids still remains a hotly debated subject, despite around fifty years of intense study. A number of different models have been proposed to explain the anti-inflammatory effect of glucocorticoids, primarily because they affect many different cell types in different ways (Whelan et al., 1995).

Administration of glucocorticoids to a patient causes a transient decrease in the circulating lymphocyte population, by redistributing the cells into body compartments (Cohen, 1972; Fauci 1975; Beardsley and Cohen, 1978), predominantly T-lymphocytes (Fauci and dale, 1974; Yu et al., 1974), and only in the recirculating portion of the intravascular lymphocytes (Fauci and dale, 1975). There is, by contrast, an increase in number of circulating polymorphonuclear leucocytes (Athens et al., 1961), reaching maximum concentration after 5 hours (Bishop et al., 1968) along with an increase in the release of neutrophils from marginated granulocyte pool, and prolonging of neutrophil half-life in the circulation (Craddock et al., 1960; Dale et al., 1974; Fauci et al., 1976). Glucocorticoids also enhance neutrophil development from precursor cells and inhibit monocyte development (Shezen et al., 1985), as well as inhibiting the adhesion of neutrophils to IL-1 activated endothelial cells *in vitro*, which could be explained by the reduction of expression of E-selectin and ICAM-1 on glucocorticoid-treated endothelial cells (Cronstein et al., 1992), which is thought to be part of the reason for the increase in the number of circulating neutrophils in the bloodstream.

Glucocorticoids also inhibit many pro-inflammatory cytokines, such as, IL-1, an important mediator of the inflammatory response, involved with induction of many other cytokines, and induction of neutrophils to the

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inflammatory site. Production of IL-1 by monocytes is inhibited *in vitro* (Snyder and Unanue, 1982), at both transcription and post-transcriptional levels (Knusden et al., 1987). The *in vivo* serum concentration of IL-1 is also reduced by glucocorticoid administration (Staruch and Wood, 1985); Expression of TNF α (Beutler et al., 1986; Beutler and Cerami, 1988), another cytokine with a central role in inflammation; The transcription of INF γ (Arya et al., 1984; Gessani et al., 1988), that activates both macrophages and neutrophils, enhancing their anti-microbial activity; GM-CSF (Churchill et al., 1992), that prolongs the survival of macrophages, neutrophils, and eosinophils, and causes an increase in expression of adhesion molecules on these cells (Lopez et al., 1986).

There are also an increasing number of other interleukins shown to be inhibited at either transcriptional or post-transcriptional levels by glucocorticoids. These include, IL-2 (Arya et al., 1984), a growth factor for T and B lymphocytes (Mingari et al., 1984), and its receptor mRNA levels (Grabstein et al., 1986); IL-3 (Culpepper and Lee., 1985), involved with immune cell development and differentiation; IL-4 (Wu et al., 1991) activator of B cells and T cell differentiation; IL-5 (Whelan, 1996) responsible for the development of, and chemotactic for eosinophils; IL-6 (Waage et al., 1993) involved with development of B and T cells, production of acute-phase proteins, and induces glucocorticoid release from adrenal cortex; and IL-8 (Kwon et al., 1993).

Biosynthesis of many of the eicosanoid lipid mediators is also inhibited by glucocorticoids (Kantrowitz et al., 1975; Lewis and Piper, 1975), perhaps due to the inhibition of cyclooxygenase enzymes, as shown with the inhibition of IL-1 induction of these enzymes in endothelial cells (Szczepanski et al., 1994). This inhibition is now thought to be via the inhibition of PLA₂ by the glucocorticoid-induced lipocortin-1 (Flower, 1988). The list of inflammatory mediators affected by glucocorticoids is

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extensive, and growing as more mediators of inflammation are being discovered, and the effect of glucocorticoids on their expression and function assessed.

1.1.8.2.) Non-steroidal anti-inflammatory drugs

Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), including methotrexate, all reduce PMN adhesion to endothelium at the site of inflammation and subsequent accumulation at these sites. Methotrexate and similar compounds are thought to act through the induction of adenosine from activated endothelial cells and fibroblasts at sites of inflammation, which reduces the adhesion of leukocytes, *in vivo* (Asako et al., 1992), and *in vitro* (Cronstein et al., 1992). Addition of adenosine deaminase reduces this inhibition. Adenosine also inhibits TNF α production (Parmely et al., 1993). It has also been shown to inhibit the superoxide generation of leucocytes *in vitro* and superoxide generation of activated leucocytes, and so may help diminish tissue damage caused by activated leucocytes at inflammatory sites.

Aspirin (Vane, 1971), and over 40 other NSAIDs in use, inhibit the synthesis of prostaglandins. Aspirin inhibition of prostaglandin is through the acetylation of CoxII, which diverts the metabolism of AA to the 15-lipoxygenase pathway producing 15 HETE which is metabolised to 14,15diHETE, a potent inhibitor of metabolic burst.

Paracetamol (non-acetylated salicylate) inhibits prostaglandin biosynthesis and reduces platelet aggregation but is not anti-inflammatory although it does reduce pain and fever.

1.1.9.) Inhibition of pro-inflammatory mediators vs. induction of anti-inflammatory mediators by glucocorticoids

Whether the main anti-inflammatory effect of glucocorticoids is to inhibit pro-inflammatory cytokines, or the induction of anti-inflammatory molecules such as lipocortin-1 is a matter for much debate. It has been proposed that an important anti-inflammatory effect of glucocorticoid is the induction of the expression of anti-inflammatory mediators. A very prominent proposed anti-inflammatory mediator that is induced by glucocorticoids is Lipocortin-1 (Flower, 1988).

The anti-inflammatory role of LC-1 has been debated for some time with contradictory evidence being presented by different research groups. Initial results indicated that LC-1 was an inhibitor of PLA₂, an enzyme that catalyses the formation of Arachidonic acid, a precursor of pro-inflammatory prostaglandins and Leukotrienes. It was shown that LC-1 inhibited the release of AA from rabbit neutrophil membranes (Hirata et al., 1980), or cultured rat renomedullary cells (Russo-Marie and Duval, 1982) but Davidson et al., 1988 indicated that lipocortin inhibited PLA₂ by binding the substrate, thus physically blocking the enzyme. This inhibition could be overcome by increasing the substrate concentration. However, it has also been shown *in vitro* to inhibit the release of lyso-PAF from rat leukocytes, the precursor of the pro-inflammatory mediator, PAF (Parente and Flower, 1985).

The possibility that Lipocortin-1 may be a second messenger in the anti-inflammatory mechanism of glucocorticoids has led to speculation that it may be used as an anti-inflammatory therapy for many of the disease states presently treated with steroids. However, the scarcity of cellular product and the instability of the recombinant protein, which spontaneously denatures *in vitro*, has meant that the approach has been to

study peptide fragments of LC-1 that have the same anti-inflammatory effects as the native protein. However, the three peptides tested for anti-inflammatory activity in *in vivo* models Ac2-26, 1-188 and antinflammin 2(246-254) have shown similar activity to the native protein, but at much greater concentrations (Perretti et al., 1993; Wei and Thomas, 1993).

1.2.) Polymorph migration stimulator. A peptide mediator of glucocorticoid effects on polymorphonuclear leucocytes

1.2.1.) Characterisation of a novel mediator of the anti-inflammatory effects of glucocorticoids

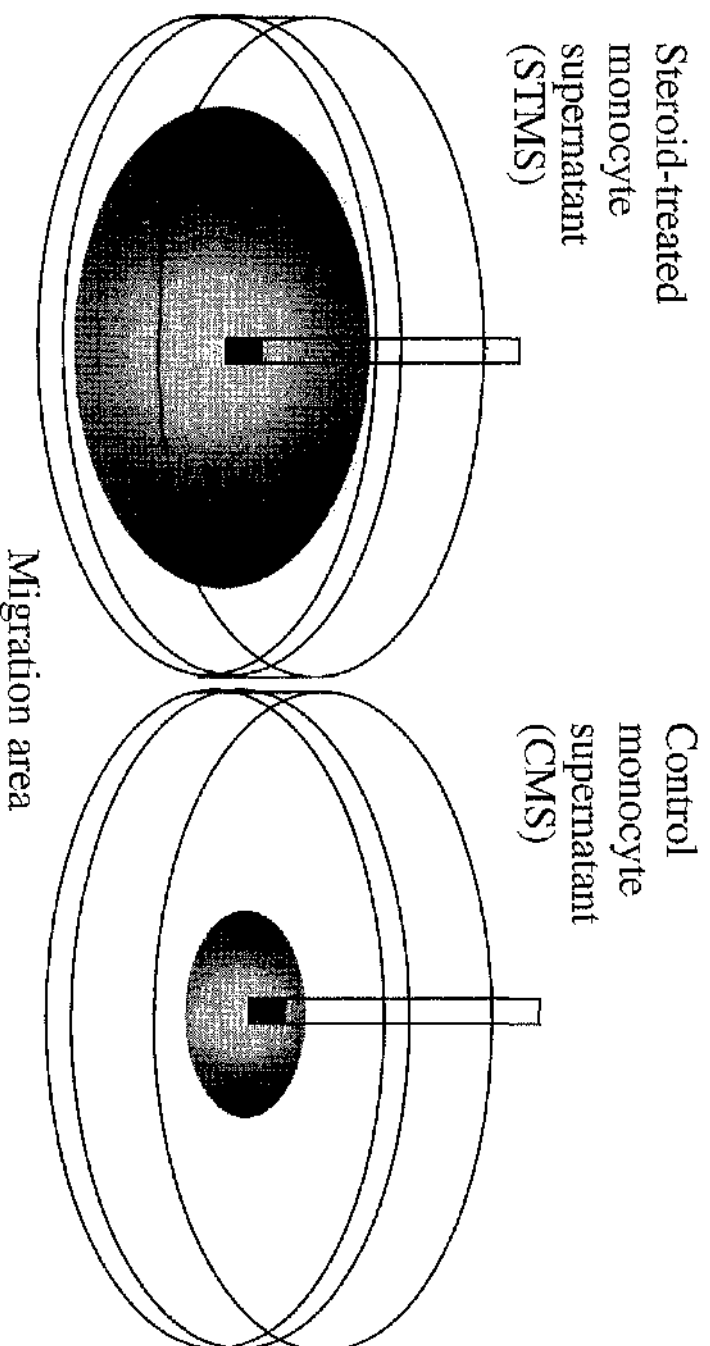
In 1974, Stevenson described a factor produced by mixed mononuclear leucocytes (MML) in response to glucocorticoids that stimulated migration of Polymorphonuclear leucocytes in a capillary tube migration assay (Fig 5). The factor(s) described by Stevenson, 1973, 1974, 1978 is the basis for this study, and a summary of his findings is below.

1.2.2.) Production of migration stimulating activity by hydrocortisone-treated mixed mononuclear leucocytes

In the capillary-tube migration assay, hydrocortisone-treated culture supernatants stimulated migration of polymorphonuclear leucocytes by a mean of 56 % compared with untreated culture supernatants and control medium with hydrocortisone (MEM-S). This activity was referred to as polymorph migration stimulator (PMS) However, it was noted that MEM-S did have some stimulatory effect on migration but shown in

Fig. 5. Schematic diagram showing the capillary-tube migration assay performed by
Dr. R.D. Stevenson

Capillary-tube migration assay



Neutrophils dispersion in presence of STMS>>>CMS

Introduction

further experiments that this was mainly due to the stimulation of contaminating mononuclear leucocytes by hydrocortisone, causing them to release the stimulatory factor during the assay. Later experiments indicated that glass-adherent monocytes were the cells responsible for the majority of the migration-stimulating activity, and showed that tissue macrophages also produce the activity. All other cell types including PMNs, platelets and lymphocytes tested did not produce significant stimulatory activity.

1.2.3.) Effect of Hydrocortisone concentration

Significant migration was shown with supernatants from mixed mononuclear leucocytes (MMLs) cultured with hydrocortisone concentrations of 0.1 μg and above. An optimal concentration of 10 $\mu\text{g/ml}$ was chosen for the majority of experiments, as the ethanol, in which the steroid was dissolved inhibited the activity at higher concentrations. It was also shown that the anti-inflammatory efficacy of a steroid directly related to its migratory stimulating capacity.

The stimulatory effect was shown with steroid at concentrations comparable to pharmacological levels in humans.

1.2.4.) Effect of MML concentration and culture duration

The stimulatory effect was not seen when MML concentration was below 10^6 cells/ml, and no significant increase in stimulatory activity was seen at MML concentrations above 2.5×10^6 cells/ml. There was an increase

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in stimulatory activity over a 24-hour period, after which no significant increase was seen.

1.2.5.) Production of migration stimulating factor by other cell types

Other cell types cultured in the presence of steroid were PMNs, platelets, lymphocytes and tissue macrophage from the spleen. The only cell type tested that produced the steroid-induced migration stimulating activity were tissue macrophages.

1.2.6.) Dose-response of steroid-treated culture supernatants

There was a fairly rapid reduction in the migration stimulating activity, coming close to that of control supernatants at a 1:8 dilution.

1.2.7.) Physical properties of the active factor from hydrocortisone-treated culture supernatants

The migration stimulating active molecule was soluble, destroyed at 90°C, and with low and high pH. Size exclusion chromatography indicated that it co-eluted with Cytochrome C, molecular weight 12400 Da. The production was inhibited by Cycloheximide, which indicated that protein synthesis was required, and the activity was destroyed by protease, further indicating the peptide nature of the active factor.

1.2.8.) Conclusions reached by R.D. Stevenson

Stevenson postulated that this factor may be a second messenger of the anti-inflammatory effect of steroids, and because it was secreted by immune cells in response to glucocorticoids, may be a possible therapeutic agent for inflammatory disorders without the undesirable side-effects of steroids. The physical properties of the active molecule indicated that it was actively synthesised in response to steroid, and is a peptide of approximate molecular weight, 12 kDa.

Frank and Roth, 1986 also described a factor(s) present in the supernatant of bovine monocytes cultured in the presence of hydrocortisone that increased the random migration of bovine neutrophils under agarose. Production of this factor(s) was inhibited by inhibition of protein synthesis and was destroyed by proteolytic enzymes indicating that the factor(s) was protein/peptide in nature. These discoveries show similarities to the factor described by Stevenson above.

With the discovery of Lipocortin-1 by Flower and Blackwell, 1979, a steroid-induced protein with potential anti-inflammatory properties, shown to inhibit Phospholipase A₂, Stevenson thought that this could be a candidate for the migratory stimulating factor he described previously. Chettibi et al. 1993 set out to see if the activity associated with PMS was due to LC-1 by using anti-LC-1 antibodies in the same capillary-tube migration assay performed by Stevenson. They found that the antibodies had no effect on the stimulatory activity of PMS. This led to a renewed attempt to characterise and purify the active factor(s) present in the steroid treated monocyte supernatant (STMS).

1.3.) Automated cell tracking assay

The parameters being measured in the capillary-tube migration assay were not clear, as the response could be attributed to increased stimulation of migration or decreased adhesiveness of the PMNs. Chettibi et al., 1993 attempted to assay STMS in a way which could distinguish more clearly these two parameters. They performed an assay developed by Dow and Lackie, 1987 which measured the movement of individual neutrophils using a computerised tracking assay. Images from a phase contrast microscope with x10 magnification were digitised and analysed by a BBC microcomputer over a period of 10 minutes. This method (Chettibi et al, 1993) showed that in a uniform concentration PMS did indeed stimulate the motility of the PMNs, and when compared with fMLP showed that the PMS stimulated cells moved in relatively straight lines. They also showed that PMS reduced adhesion of PMNs to bovine serum albumin (BSA) coated coverslips when compared to control monocyte supernatants and culture medium with steroid. Anti-IL-8 antibodies had no effect on the migration stimulation. PMNs from patients under steroid therapy were also more motile than cells from control patients. However, the parameters calculated in these assays, namely speed and persistence showed no differences between the two, despite a clear difference upon visual inspection of the tracks, so it was clear that the data processing in this assay was limited in discerning different types of cellular locomotion.

A new assay was developed which used a phase contrast microscope at x32 magnification. A video camera was attached to a Acorn A5000 computer with digitiser, and a computer program was written by P. Lawrence to measure cell motility and provide accurate data for

calculation of the parameters of locomotion described by Gail and Boone, (1970, 1972), namely speed, diffusion coefficient and persistence.

1.4.) Purification chromatography techniques

The techniques utilised in a purification protocol are very dependent on the characteristics of the molecule being dealt with, namely its size, charge and hydrophobicity. The material from which it is being purified is also crucial, be it biological tissue, bodily fluids, or culture supernatants. The initial stage of purification is determined by the volume of starting material, as most standard chromatography techniques can only deal with volumes up to 10's of ml, so a suitable concentration method must be found before starting the purification, in order to allow standard chromatography techniques to be utilised. All these things have to be considered before deciding on which techniques will be used in purification of the active molecule in the starting material.

The purification attempts of Dr. Chettibi indicated that the active molecule, present in the starting material, now known as steroid-treated monocyte supernatant (STMS), was in fact a relatively small molecule, around 1-2 kDa. The charge of the molecule was uncertain as it behaved inconsistently throughout various purification attempts, binding to MonoQ, an anion exchange support at pH 8, early in the purification protocol, but apparently failing to repeat this at later stages. The hydrophobic nature of the molecule was also uncertain, as it appeared at early stages to bind partially to a phenyl sepharose column, with activity being found in both the bound and unbound eluates. However, the active molecule did seem to bind quite consistently to a reversed-phase Sephasil

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C8/C18 column. The activity was however, lost on a regular basis during purification, for unknown reasons.

The fact that the starting material was cell culture supernatant with very little serum protein present helped the purification, as it contained relatively small amounts of contaminants. The active molecule was apparently present in very small quantities, making it very important to reduce to a minimum the number of steps in the purification protocol, as each step usually gives around 75% or less yield, so the quantity of active material would be reduced to $< 30\%$ after 4 steps. Certain types of column were chosen to attempt purification of the active molecule in STMS. A Pharmacia Superdex 30 which has a separation range of up to 10000 MW, was used to separate the active material from large proteins and eluted the activity just before the salt.

Due to the requirement to reduce losses of active material during the purification, it was important to be able to elute the activity from columns in as small a volume as possible, whilst maintaining good separation. The HPLC system that was employed was the Pharmacia SMART system, which had been specifically designed for micro purification. The columns that were available for the SMART system were of very small diameter and small bead size giving high quality separation and small elution sample size. The system has very small dead volumes, which reduces dilution and improves accuracy in the collection of samples, and was able to collect samples as small as 5 μ l. The high sensitivity of this system also allowed detection of peaks containing < 1 ng. Concentration of samples after chromatography techniques is often required if the activity is spread over several samples. This was achieved using a vacuum centrifuge (Warren Scientific), which allowed the concentrating of samples from 2-3 ml down to around 20 μ l. This method of drying samples has the advantage of keeping the sample centrifuged to

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the bottom of the tube reducing loss when removing the sample. This is very useful when changing from an ion-exchange or reversed-phase column to a size exclusion chromatography column, where a the smaller the injected sample the better the separation tends to be.

1.5.) Characterisation and purification of the active factor in STMS

Stevenson, 1978 made a number of discoveries about the physical characteristics of the active molecule present in his culture supernatant. He showed that the migration stimulating active molecule was soluble, destroyed at 90°C, and at low and high pH. Size exclusion chromatography indicated showed that it co-eluted with Cytochrome C which has a molecular weight of 12400. The accuracy of this technique and conditions of running only gave a rough indication of the size of the molecule (Dr. Stevenson; personal communication). He also showed that the production was inhibited by cycloheximide, which implied that protein synthesis was required. The activity was also destroyed by protease.

These results indicated that the molecule was most likely peptide in nature, of size in the region of 3000 - 15000 MW. The quantity present in the supernatant was undetermined and the nature of the activity of the purified molecule was unknown, i.e. It was unclear if the activity was a single molecule, or if it maintained the same level of activity following purification.

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1.6.) Characterisation and purification of the active molecule in STMS by Dr. S. Chettibi

Dr. Chettibi attempted the purification of the active factor in STMS starting material. He approached the problem by trying various ways of concentrating the initial starting material to a volume which allowed ease of handling during further purification steps. Starting with a 300 ml batch of STMS for each purification attempt he utilised ultrafiltration techniques, with various molecular weight cut-off filters from Amicon and Millipore filtration systems. Unfortunately this technique seemed to produce an inconsistency in the activity, i.e. activity was found in the retained and eluted portions. The activity also came through the smallest available molecular weight cut-off filters of 500 Da. This was therefore not an appropriate concentration method.

He then tried using prep grade Bio-Rad Q, an anion exchange matrix. Unfortunately the activity would only bind under low salt conditions, and so required a ten-fold dilution. This meant that around 3 litres of liquid had to be pumped through the MonoQ column which took a considerable time. Bacterial/fungal contamination became a major problem. The activity eluted from the MonoQ column at around 150 mM sodium chloride when a 0-500 mM sodium chloride gradient was used. This produced the active factor in a volume of around 10 ml. Subsequent gel filtration on a Pharmacia Sephadex G30 showed that the activity eluted in the region just before the salt, with another active region associated with larger proteins. This indicated that there may have been two active factors present in the supernatant, one a large ~60 kDa and another, perhaps a fragment of the larger of around 1 kDa. The smaller of these two molecules was the most active and interesting, and in consequence became the subject of further purification attempts.

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The active samples were then applied to a reversed-phase chromatography column. The eluted peaks were pooled into glass tubes and freeze-dried. They were then re dissolved in 1ml of water and assayed at 1/10 in Hank's HEPES v/v. This yielded an activity similar to that of starting material. When this sample was applied to Malдитof (Matrix assisted laser desorption time of flight) mass spectroscopy (Prof. Rammage, Edinburgh), it showed a strong peak of mass 1328 (Fig 6). Sequencing attempts of this molecule were wholly unsuccessful, and it was inferred that if the 1328 molecule was a peptide, it must have a blocked N-terminal. Applying this sample to the Pharmacia peptide column showed however, that the main UV absorbing peak from the reversed-phase column was in fact running as a very small molecule, co-eluting with the salt fractions at V_t (not shown), with minimal UV absorption at the elution point of a molecule of molecular weight, 1328.

Fig. 6. Mass spectroscopy performed on an active sample from a reversed-phase chromatography column

Malditof (Matrix assisted laser desorption time of flight) mass spectroscopy (Prof. Rammage, Edinburgh), showing a strong peak of mass 1328.91 in the active sample from the reversed-phase chromatography column of Dr. Chettibi. The two higher mass peaks correspond to the sodium and potassium adducts of the native molecule.



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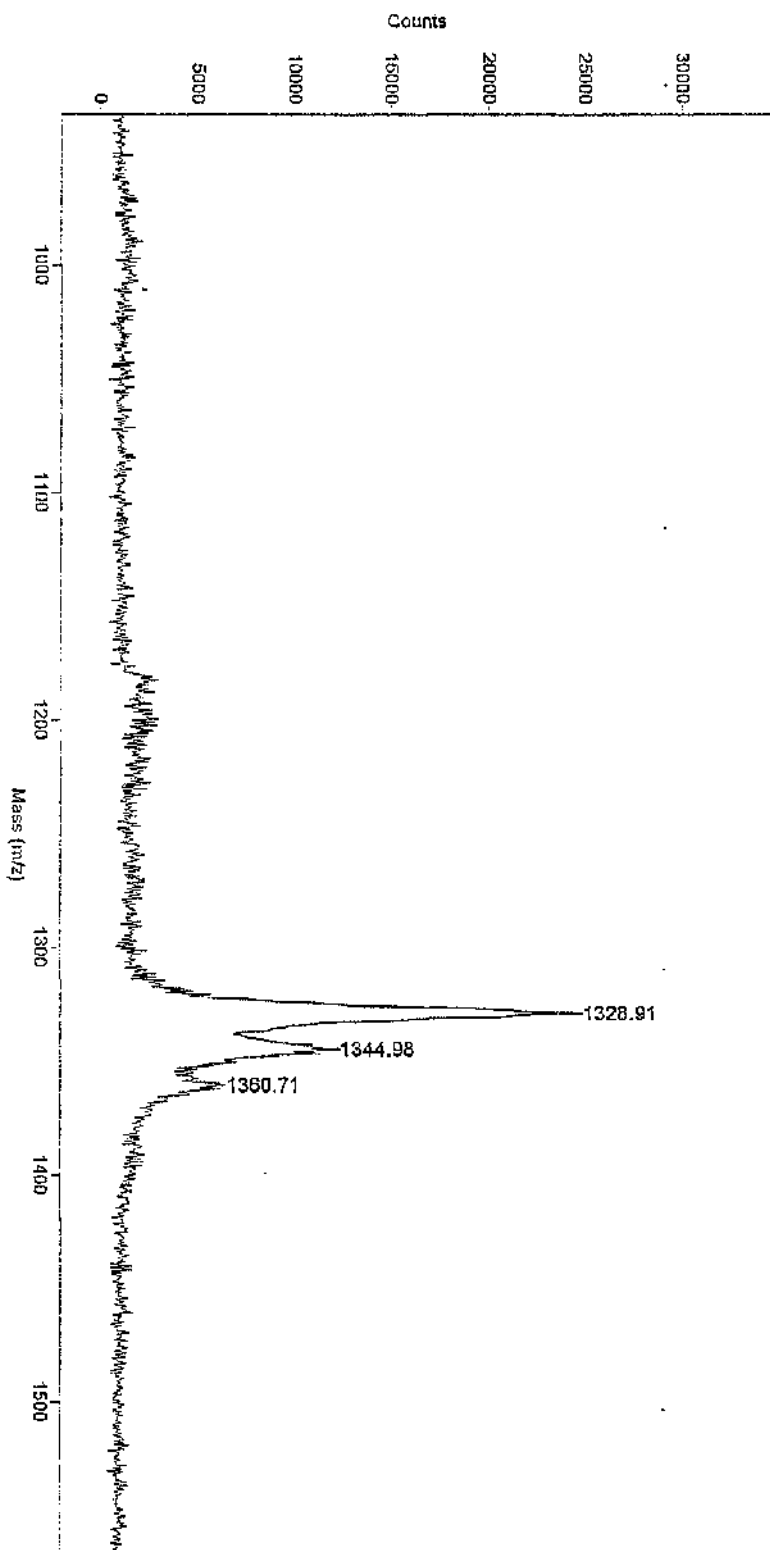
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Sample: 67



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Laser: 685

Low Mass Gate: OFF

Scans Averaged: 4

Negative Ions: OFF

Pressure: 1.84e-07

MATERIALS AND METHODS

2.1.) Materials

2.1.1) Interleukin-8 (IL-8) (Genzyme), dissolved in phosphate buffered saline (PBS) and stored in 50 μ l aliquots at 20 μ g/ml in -70°C.

2.1.2.) Lysophosphatidic acid (1-oleoyl) (LPA) (Sigma, Chemical Co., Dorset) was made up at concentration of 3.5 mg/ml in ethanol: chloroform (1:2 v:v).

2.1.3.) n-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma) was made up as a 10^{-2} M stock solution in Dimethyl sulfoxide (DMSO), and stored in 100 μ l aliquots at -20 °C.

2.1.4.) Phorbol 12-myristate 13-acetate (PMA) (Sigma) made up at a concentration of 1 mg/ml in DMSO.

2.1.5.) 7-dimethylamino-naph-thalene-1,2-dicarboxic acid hydrazide (DNDH) (Boehringer) was made up at a concentration of 3 mg/ml in DMSO.

2.1.6.) Pertussis toxin from *Bordetella pertussis* (Sigma) was used after dilution in PBS, pH 7.4.

2.1.7.) TNF α (Genzyme), was dissolved in phosphate buffered saline (PBS), aliquoted into 100 μ l samples, and stored at 1 μ g per ml at -70 °C.

2.2.) Buffered saline solutions

Several different buffered saline solutions were used throughout this study:

2.2.1.) Balanced Salt Solution (BSS)

A two-part mixture was made up to 1 L (at 10x strength)

1. 10g sucrose (Fisons scientific equipment, Loughborough, Poole, England)
0.6g Potassium dihydrogen orthophosphate (BDH chemicals, Poole, England).
3.58g disodium hydrogen orthophosphate (BDH).
2. 1.86g Calcium chloride (Fisons).
4g Potassium chloride (Fisons).
80 g Sodium chloride (Fisher scientific, UK).
2g Magnesium chloride.6H₂O (BDH).
2g Magnesium sulphate (BDH).

Parts were mixed 1:1 (v:v) and made up to 1x concentration by adding water.

2.2.2.) Phosphate Buffered Saline (PBS)

8g Sodium Chloride,
0.2g Potassium Chloride,
1.15g Disodium Hydrogen Orthophosphate and
0.2g Potassium Dihydrogen Orthophosphate were dissolved in water, and made up to 1L before sterile filtering into aliquots.

2.2.3.) HEPES Water (HO)

5.25g HEPES (Sigma) was dissolved in water, the pH adjusted to 7.4 using 1M HCl, made up to 1 Litre, and dispensed into aliquots before autoclaving and storing at 4 °C.

2.2.4.) HEPES Saline (HS)

2.38g HEPES,
1g Glucose (BDH),
8g Sodium Chloride and
0.4g Potassium Chloride were dissolved in water, the pH adjusted to 7.4 using 1 M Hydrochloric acid (HCl) (Prolabo), made up to 1 Litre with water, and sterile filtered into aliquots before storing at 4°C.

2.2.5.) Hank's HEPES (HH)

As HS but containing 1mM Calcium Chloride and Magnesium Chloride.

2.3.) Antibiotics

A solution of L-Glutamine (29.2 g/L) was made up in water, and sterile filtered. A solution of Streptomycin Sulphate (76.2 mg/L) was also made up in water. 150 ml (200 mM) Glutamine solution, 100 ml Streptomycin Sulphate and 12.5 ml Amphotericin B were mixed, aliquoted aseptically and kept frozen until use.

2.4.) Ham's F-10 culture medium

20 ml of 10x strength Ham's F-10 culture medium (GIBCO) was added to 180 ml of HEPES water. 1 ml of 7.5% bicarbonate solution (w/v) was added. If serum was required then 20 ml of foetal calf serum was added.

2.5.) Dexamethasone preparation

Dexamethasone (Sigma) was freshly made up at a concentration of 10^{-2} M in ethanol and added to the monocyte culture medium at a final concentration of 10^{-6} M dexamethasone and 0.001% ethanol. The control culture medium also contained the same concentration of ethanol.

2.6.) Cell preparation and culture

2.6.1.) Preparation of human Neutrophils

Human neutrophils were purified by a modification of the method of Haslett *et al.* (1985). Whole blood from healthy volunteers was mixed in a ratio of 1:10 (v:v) with 5% Dextran MW 500,000 (Sigma) and left at 37°C for erythrocyte sedimentation to occur. The leucocyte-rich buffy coat layer was then aspirated and layered over a nyco-prep gradient density, Nycoprep 1.077 (Nycomed), and centrifuged at 800 rcf for 15 minutes at room temperature. The resulting pellet consisted of polymorphonuclear leucocytes and some contaminating erythrocytes. The remaining erythrocytes were removed by hypotonic lysis in distilled water for around 20 seconds, and the purified polymorphonuclear leucocytes were washed three times (1500 r.p.m. for 5 minutes at room temperature) in HH and re suspended in the same buffer at a

concentration of 10^6 cells ml^{-1} (Fig. 7). Cells prepared by this method were $> 95\%$ neutrophils. Viability was tested by trypan blue exclusion and was found to be 98-99%.

2.6.2.) Isolation of human monocytes

Two sources of human monocytes were used.

2.6.2.1) Isolation and culture of human monocytes from whole blood

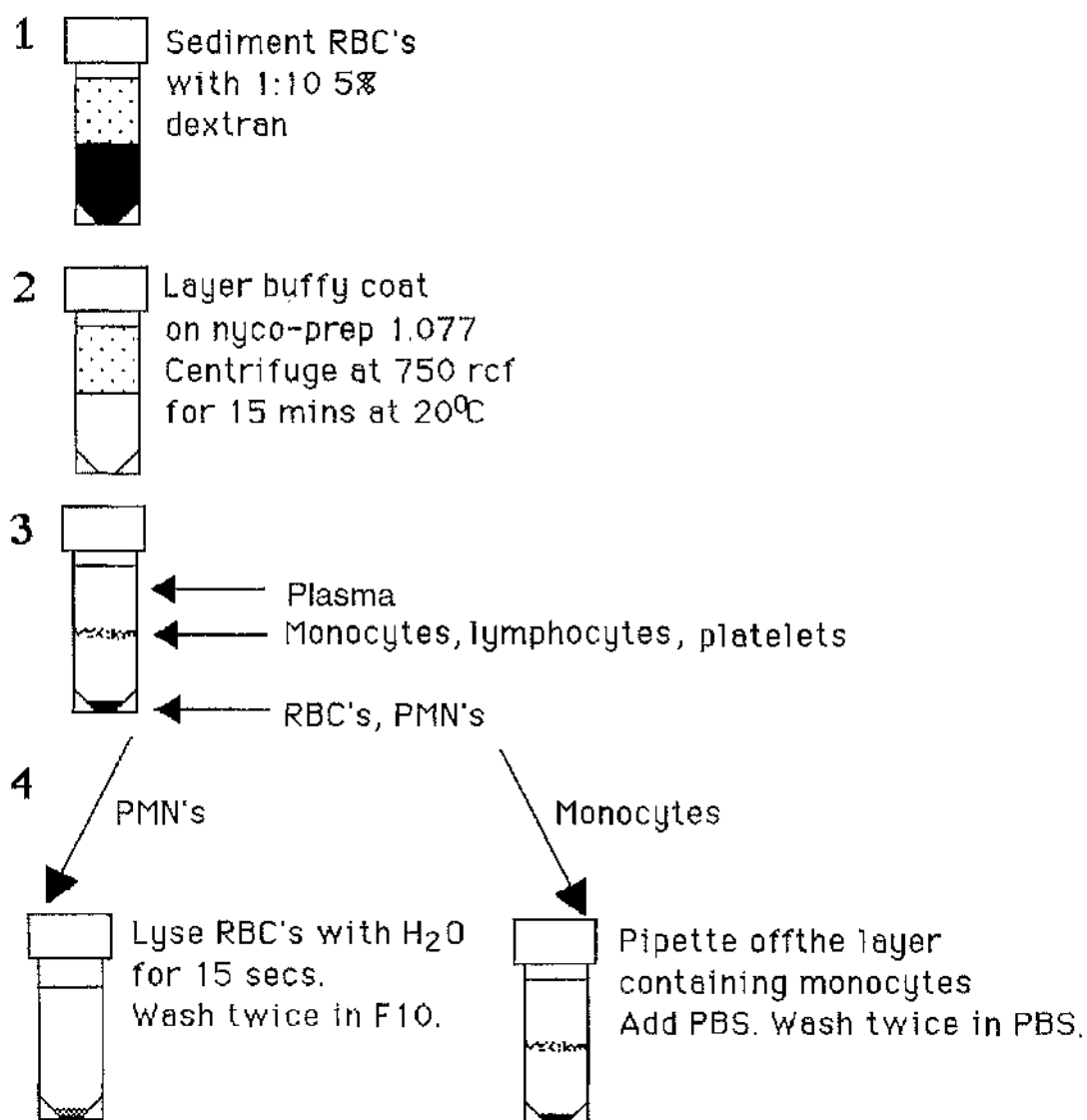
Units of whole blood received from the Royal infirmary in acid-citrate dextrose anti-coagulant were mixed 1:10 with 5% dextran of average molecular weight, 500,000 in HS for 1 hour which allowed most erythrocytes to sediment. The leucocyte-rich plasma was then aspirated and layered over Nycoprep (Nycomed) 1.077, centrifuged 800 rcf for 20 minutes at room temperature. The mononuclear leucocyte layer was then aspirated. These cells were then washed twice with HEPES saline (300g 4 min), which removed most of the contaminating platelets (Fig. 7). They were then cultured in HEPES buffered Ham's F10 with 10% Foetal calf serum (FS) and antibiotics, for 1 hour, washed twice with HEPES saline warmed to 37°C , then cultured for 24 hours in serum-free Hams F10 with and without 10^{-6}M dexamethasone. The conditioned culture medium was then centrifuged at 3660 rpm for 20 minutes at room temperature and sterile-filtered through a $0.22\ \mu\text{m}$ filter (Millipore). This was the starting material for purification.

2.6.2.2) Isolation of human monocytes from leucocyte-rich buffy coats

Leucocyte-rich buffy coats were supplied by the Blood transfusion service. This was layered over a Nyco-prep gradient density in a 50:50 ratio (v/v), and centrifuged at 800g for 20 minutes at room temperature. The mononuclear leucocyte layer was then aspirated and treated as monocytes cultured from whole blood.

Fig. 7

Purification of PMN's / Monocytes from human blood



Materials and Methods

2.6.3.) Techniques, solutions and culture medium used in the preparation of Bovine aorta endothelial cells:

2.6.3.1.) Saline solution

150 mM Sodium chloride containing antibiotics.

2.6.3.2.) Collagenase solution

150 ml Dulbecco's modified eagles medium (DMEM) with sodium pyruvate, 1.25 mg/ml collagenase.

2.6.3.3.) Bovine endothelial cell culture medium

DMEM with sodium pyruvate containing antibiotics, 10% Foetal calf serum and 10% Horse serum.

2.6.3.4.) Trypsin / Versene

Versene was made up essentially as for HS except that EDTA (Sigma) was added at a concentration of 0.2g/L before adjusting the pH to 7.5. The versene was then dispensed into 20 ml aliquots prior to autoclaving and storage at 4°C. Immediately before use.

Immediately before use 0.5 ml of sterile 2.5% solution of trypsin (GIBCO) was added to the versene and mixed.

2.6.3.5.) Preparation of Bovine endothelial cell monolayer

Aortas collected from the local abattoir were flushed through thoroughly with saline solution to remove any blood from inside and most of the outer fat was cut off. The large end of the artery was then tied off and saline solution was injected before returning to the laboratory. The remaining fat was then carefully cut away from the outer vessel wall and the intercostal arteries were tied off with thread. 20 ml of collagenase solution was injected and the artery wrapped in cling-film and incubated at 37 °C for 15 mins. After incubation, the aorta was massaged gently to displace endothelial cells from the inner wall and the solution containing the cells was collected. The cells were washed twice with culture medium centrifuging at 300g, 5 °C for 6 minutes. The washed cells were re-suspended in 20 ml of cell culture medium and grown in 50 cm² flasks at 37 °C in a humidified, 5% CO₂ to 95% air atmosphere.

2.6.3.6.) Passaging of Endothelial Cell Cultures

When the cultured endothelial cells reached confluency they were passaged and recultured to maintain their growth. The culture medium was poured off and the cell monolayer washed twice with HS to remove all serum from the flask. 3 ml of Versene/trypsin solution was added to the culture and incubated for 1 minute at 37 °C. The versene/trypsin solution was then poured off and the cells were left at 37 °C for 5 minutes, or until they detached. 5 ml of culture medium containing serum was added, and suspension triturated to remove any cells still attached. Cells were washed twice, by spinning at 600 g. for 5 minutes at 4 °C. Cells were counted in a haemocytometer and recultured in 50 cm² culture flasks with 5 million cells in 20 ml of culture medium at 37 °C in

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humidified 5% CO₂ to 95% air atmosphere. These flasks gained confluency in approximately 5 days. Cells which were not required immediately were stored for later use by cryopreservation.

2.6.3.7.) Cryopreservation of endothelial cells

Growth medium was removed, and the cells washed twice with HEPES saline. 3ml of Versene/trypsin solution was added to the culture and incubated for 1 minute at 37 °C. Excess trypsin/versene was poured off, and cells left for 5 minutes, or until detached. 5 ml of culture medium containing serum was added, and suspension triturated to remove any cells still attached. Cells were washed twice by centrifuging at 1500 rpm for 5 mins. After the second spin the cells were re suspended in 10% DMSO, at concentration of around 5 million/ml, and aliquoted into sterile cryotubes. Cells were stored at -70 °C.

2.6.3.8.) Recovering cryopreserved cells

To recover cryopreserved cells, the cryotube was defrosted in a 37 °C water bath. The cell suspension was then added to 10 ml of DMEM culture medium, washed by centrifugation at 1500 rpm for 5 minutes, and re suspended in fresh culture medium. Cells were cultured in a humidified 5% CO₂ (BOC) 95% air atmosphere.

2.6.4.) Trypan blue exclusion

The viability of prepared leukocytes was assayed using Trypan blue exclusion. Cells were diluted 1:10 in 0.05% trypan blue and the

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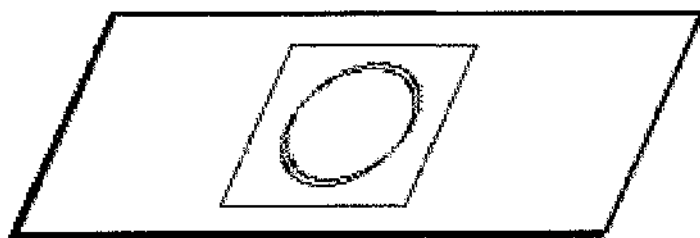
percentage of viable cells was determined by counting the number of cells excluding the dye, in a haemocytometer.

2.7.) Neutrophil automated cell tracking assay

2.7.1.) Preparation of migration chambers

A stainless steel slide 75 x 35 x 1 mm with a 18 mm diameter circular aperture was heated under flame and a 22 x 22 mm square glass coverslip was fixed over the aperture using hot paraffin wax. The chamber was then turned over, and the glass surface was coated in Bovine serum albumin (BSA) by adding 0.5 ml of 0.5% BSA in water (w/v) for 20 minutes. The coverslip was then washed once with HH and 0.2 ml of neutrophil cell suspension at 10^6 cells/ml was added and left for 10-15 minutes, for the neutrophils to adhere. The non-adherent cells were removed by washing the well with HH, and 0.3 ml of the treatment of interest was added to the chamber. A second coverslip was then placed over the chamber, and the edges sealed by brushing over with hot paraffin wax (Fig. 8).

Fig. 8. Schematic diagram of tracking assay migration chamber



2.7.2.) Modified tracking assay

Migration chambers were placed on the stage of an inverted microscope within a temperature controlled (37 °C) transparent box, and locomotion observed using a x32 objective optic, by means of a video camera connected to a monochrome monitor and an Archimedes A5000 computer with a Watford video digitiser, programmed to capture and analyse one frame every 5 seconds.

2.7.2.1) Automated cell tracking

The tracking program selected up to 80 cells, in rank order of their distance from the screen centre. Selection was based on finding an island greater than a threshold area, composed of pixels above threshold brightness. The program display was designed to reveal the tracking status of each target cell; firstly, the cells were displayed in 16 grey shades, each active target cell was outlined in red and the line joining its centre to the previous recorded position was plotted. The cells were then unplotted, leaving the outlines of the target cells, which were then unplotted to leave only the cell tracks. For each frame the computer

stored the x and y co-ordinates of the centres, the perimeter length and the enclosed area of the target cells.

A program running at the required speed was written in C and was readily subject to modification and refinement of data processing. Target cell searches were carried out synchronously by advancing one pixel for each cell, thus giving priority detection to cells that had moved the shortest distance, rather than an asynchronous search where each search box was completed in turn, thus giving priority detection to cells in rank order (P.D. Lawrence, unpublished work). Visual inspection of both processes showed that the tendency to assign cells incorrectly increased much more rapidly with search-box size for asynchronous than for synchronous searches. This clearly reflects the fact that the cells that can be assigned with greatest certainty are those that have moved the shortest distance. Numerical simulation can be used to confirm the supposition that by assigning targets in order of distance moved, the synchronous search minimises wrong assignment of the least certain target cells.

2.7.2.2.) Target cell loss

Target cells were lost to the tracking assay for a variety of reasons. For highly motile cells, these include, moving beyond the search box, touching the screen edge and touching another cell. In addition some stimuli activate a reversible spreading process that causes large parts of the cell to become phase-dark, resulting in target loss. Such cells are not recovered as targets when they re-brighten. In principle, each of these mechanisms should apply to different extents with the different stimuli, but analysis of a large body of data did not reveal any significant correlation between the rate of target cell loss and either the speed or

dispersion rates. Neither did the stimuli that appeared to be most active in terms of cell shape-change induction give significantly enhanced loss rates.

2.8.) Chemotaxis assay

Chemotaxis assays were performed in a modified Boyden chamber. This consisted of the cut-off barrel of a 1 ml syringe with a 3µm pore filter glued to the bottom of this upper chamber. 200 µl of the upper chamber assay solution (antagonist) containing 1×10^6 /ml of human neutrophils, was placed in the upper chamber. This chamber was then placed into the lower chamber, which consisted of a 5 ml glass beaker with 4.2 ml of agonist (Fig. 9). These were then incubated for 90 minutes at 37 °C. The cells were then fixed in 70% ethanol for five minutes. This treatment also dissolved the glue allowing the filters to be removed by a fine set of forceps. The filters were then washed in distilled water for 3 minutes, then the cells were stained using Harris haemotoxylin for 5 minutes. The colour was then developed, following a further wash in distilled water for 3 minutes, using Scott's tap water (equal volumes of 0.7% sodium bicarbonate (w/v) (Sigma) and 4% Magnesium sulphate (w/v)) for 5 minutes. The filters were then dehydrated by placing in an increasing concentration of ethanol 70%, then 95% and finally in 80% ethanol: 20 butan-1-ol for three minutes each. The filters were made transparent by placing them in 100% xylene for 5 minutes, then mounted in Depex on a glass slide. The slides were then left on the bench to dry before the chemotaxis could be measured.

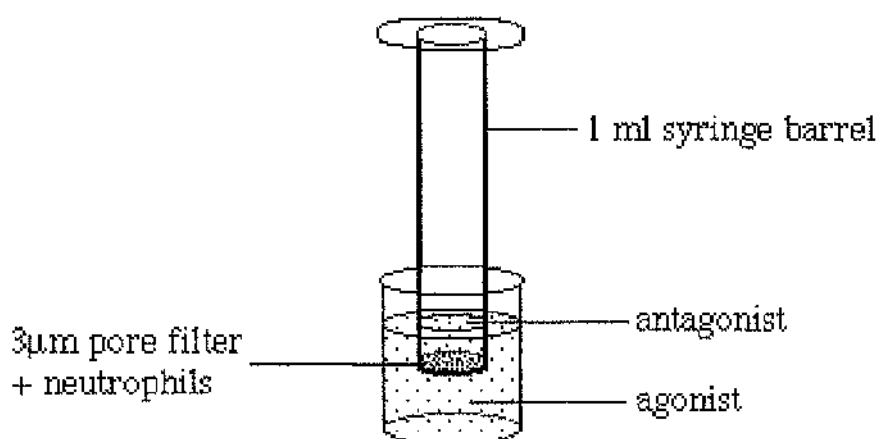
Measurement of chemotaxis: Analysis of neutrophil chemotaxis was performed on an inverted microscope. Two methods were employed for this analysis; the measurement of the leading front, and the number of

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cells that had moved half the leading front distance of the positive control. The leading front method of analysis measured the distance that the two furthest cells had moved into the filter. This was done by focusing on the cells sitting on top of the filter and noting the micrometer reading on the focus. The microscope was then focused into the filter until the last two cells came into focus, and the reading on the micrometer was noted. The difference in these two readings gave the leading front distance. Five readings were taken for each filter in randomly chosen fields, and there were three repeats of each assay. Whilst performing this analysis it was noticed that under certain conditions a very small number of cells were moving far into the filters and giving an apparently positive result i.e. in the positive control many cells moved into the filter but this type of analysis only showed a relatively small difference. In order to compensate for this, a second method of analysis was employed. The distance of the leading front in the positive control was measured and the number of cells that had moved half that distance was counted for each of the treatments. Again, five randomly chosen fields were analysed for each filter. This analysis gave a much more representative result, confirming in the data, the obvious differences between the treatments observed under the microscope.

Fig. 10

Chemotaxis modified Boyden chamber



2.9.) Chemiluminescent measurement of neutrophil respiratory burst

Metabolic activation of neutrophils was assayed by 7-dimethylamino-naphthalene-1,2-dicarboxylic acid hydrazide (DNDH)-enhanced chemiluminescence using an LKB 1251 Luminescence Photometer (Luminometry; LKB Wallac Oy, Turku, Finland). Neutrophils in HH, at a final concentration of 1×10^5 cells/ml, were stimulated by either fMLP or PMA at concentrations of 1×10^{-5} M, and 10 ng - 1 µg/ml, respectively. Data was collected continuously using an Acorn Archimedes microcomputer, with software written in C (Appendix II), which displayed the results in the form of a line graph, and calculated the area under the graph for each assay sample.

2.10.) Neutrophil adhesion assay

Bovine aorta endothelial cells were cultured on 13 mm diameter glass coverslips in a multi-well dish in DMEM with 10% foetal calf and 10% horse serum and grown to confluence. Human neutrophils suspended in 0.1% bovine serum albumin (BSA) in HH (w/v) were labelled with by incubating them at 1×10^6 cells/ml for 1 hour with 20 $\mu\text{Ci/ml}$ (^{51}Cr) sodium chromate. Free ^{51}Cr was removed by three washes with HH 0.1% BSA. 200 μl of neutrophils were mixed with 800 μl of test substance, added to the wells and incubated for 30 minutes at 37 °C. Non-adherent cells were washed three times with HH 0.1% BSA, and coverslips were placed in a Wilj gamma counter. There were three repeats for each experiment.

2.11.) Analysis of F-actin content of neutrophils

Purified neutrophils were placed on albumin coated glass coverslips, washed to remove non-adherent cells then treated with various stimuli for 30 minutes at 37 °C. The cells were then fixed using 1% paraformaldehyde in serum-free Ham's F-10, for a further 30 minutes, and permeabilised with 1% Triton X-100 for 15 minutes at room temperature. Cells were gently washed three times with HH and treated with 0.1 $\mu\text{g/ml}$ TRITC-labelled phalloidin (Sigma) for 20 minutes at room temperature. Cells were washed three times with HH, being left for 5 mins between washes to allow the unbound phalloidin to diffuse from the cells, then the coverslips were mounted onto glass slides in 50% glycerol in water (v/v). Results were analysed using an Odyssey confocal microscope.

2.12.) Purification techniques

2.12.1.) Concentration of starting material

Two techniques were used to concentrate the starting material to more convenient volumes

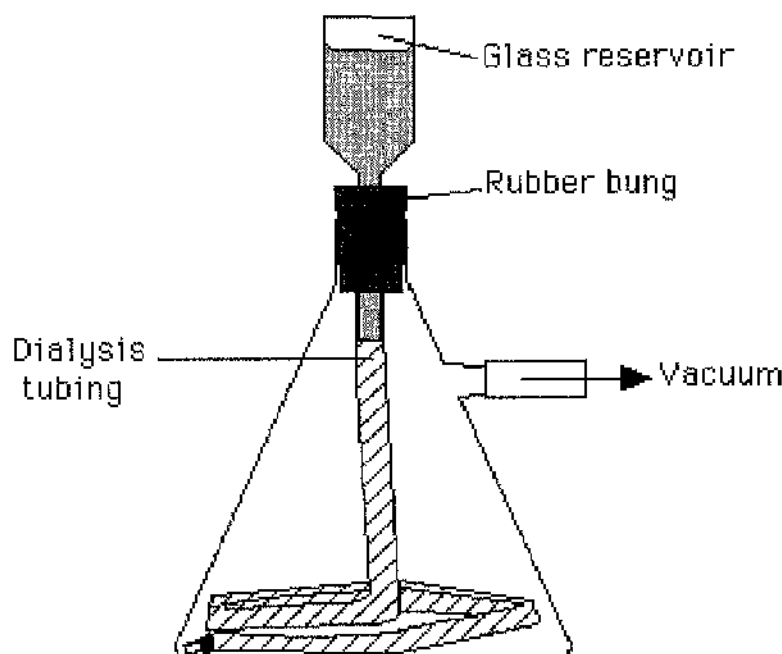
2.12.1.1.) Freeze-drying

Prior to initial purification steps around 600 ml of starting material was freeze-dried in an Edward's high vacuum limited speedi-vac ED150 100 ml, to a volume of around 20ml. This solution was then centrifuged at 3660 rpm for 20 minutes to remove denatured protein and precipitated salts, before being filter sterilised through a 0.22 μ m filter.

2.12.1.2.) Force Dialysis

The starting material was force dialysed against air, using Visking dialysis tubing, size 1; Diameter 8/32"-6.3 mm with molecular weight cut-off of 12-14000 Da (MEDICELL International Ltd., London). To facilitate dialysis of large volumes 2 metres of Visking tubing was used and a glass reservoir was topped up at regular intervals (Fig 10).

Fig. 10. Schematic diagram of vacuum dialysis apparatus



2.12.2.) Chromatography Techniques

2.12.2.1.) FPLC chromatography

A Pharmacia XK50/500 column was filled with swollen Bio-gel P2 size exclusion chromatography gel matrix (Bio-Rad), and 150 mM sodium chloride was used as running buffer. This was run at a flow rate of 1.5 ml/min. Approximately 40 ml samples were loaded at a time, by replacing the buffer supply tube into the sample. A Bio-rad Econo system was used for these analyses, detection was at 254 nm and the output was on a Bio-rad pen-recorder. Samples were run in 150mM Sodium chloride to prevent ionic interactions between molecules in the sample and the gel matrix.

2.12.2.2) SMART System

HPLC purification was performed on the Pharmacia SMART system, using mainly Pharmacia chromatography media and columns. The SMART HPLC system is used for micro-purification, giving good sample recovery efficiency, due to its small void volume (17 μ l). It is controlled using SMART system software, running on a Compaq Deskpro microcomputer with OS2/Warp operating system. Methods for separation can be programmed through software control and data collected continuously, displayed on-screen and stored for further analysis. Data analysis is possible by peak integration, giving areas under elution peaks, thus allowing comparison of different separations, and quantitation of peaks by comparison to standards.

Absorbance was measured at 214 nm during all chromatography on the SMART HPLC system for this study. A 1 mg/ml concentration of a standard protein e.g. BSA, gives an absorbance of 1.0 AU, at 280 nm and 1.0 AUFS. At 214 nm the level of absorbance of proteins is around 15 x that of 280 nm absorbance. This wavelength was chosen because of the increased sensitivity given, and our lack of knowledge about the molecule that we were attempting to purify. If it was peptide in nature, then to provide adequate absorbance to allow detection at 280 nm, it would have to contain a number of amino acids that absorb well at 280 nm; tryptophan and tyrosine.

All reagents used were either of Analytical or HPLC quality.

2.12.2.2.1) Superdex peptide

Superdex peptide HR 10/30 consists of a composite of cross-linked agarose and dextran with bead diameter of 13-15 μm . It has an exclusion limit of 20 kDa, with optimal separation in the range of 0.1 - 7 kDa. The bed volume is 24 ml, with a maximum flow rate of 1.2ml/min, pressure limit of 1.5 MPa, and pH stability of 1-14.

Injected samples were between 20-250 μl , and flow rates used were 500 $\mu\text{l}/\text{min}$. Calibration showed that V_0 was approximately 10ml and V_t , 20ml. Fractions of 500 μl were collected from V_0 - V_t .

Running buffer consisted of either 1-10 mM Hydrochloric acid in 20% (v/v) Acetonitrile (Fisher scientific), or 0.1% (v/v) Trifluoroacetic acid (TFA) (Sigma) in 20% acetonitrile. The acidic conditions were to prevent ionic interactions between molecules in the samples and the gel matrix. Eluates were collected at 500 $\mu\text{l}/\text{sample}$ in 0.6 ml eppendorf tubes, and stored at -20 $^{\circ}\text{C}$.

2.12.2.2.2) Ion-Exchange chromatography

Pharmacia Mono Q HR 5/5 is a strong anion exchanger, with a composition of hydrophilic resin with charge group $-\text{CH}_2-\text{N}^+(\text{CH}_3)_3$. It has pH stability in the range 2-12. A 20mM Tris.Cl buffer was used, and samples eluted with a 0-500 mM Sodium chloride gradient, over 15 minutes.

Buffers: A. 20mM Tris.Cl pH 8.1

B. 20mM Tris.Cl 500 mM NaCl pH 8.15

The differences in pH of the two buffers was to correct for UV absorbance differences between the low and high salt solutions, thus

providing a level baseline during gradient formation. Eluates were collected at 500 μ l/sample in 0.6 ml eppendorf tubes, and stored at -20°C.

2.12.2.2.3.) Reversed-phase chromatography

A Pharmacia Sephasil C18 4 x 250 mm reversed-phase column was used for high performance separation of samples. This is composed of a Silica base matrix, with a particle size of 5m, and pore size of 100-200 Å. It has temperature stability in the range 4-75 °C, pH stability 2-10, a pressure limit of 35 MPa/3500 psi, with recommended pressure ceiling for use, and recommended flow rate between 0.5-1.0 ml/min. A 0.1% TFA buffer was used, and samples eluted with a 0 - 50% Acetonitrile gradient, over 30 minutes. Eluates were collected at 100-250 μ l/sample in 0.6 ml eppendorf tubes, and stored at -20 °C.

Buffer A. 0.14% TFA (v/v)

 B. 0.12% TFA (v/v) 50% acetonitrile (v/v).

The difference in the TFA concentration of buffers corrected any differences in the UV absorbence of the two solvents, thus providing a level baseline during gradient formation.

2.12.2.3.) Purification of Thymosin β 4 from Human Blood Buffy coats

Thymosin was purified using the technique of Hannappel and van Kampen, 1987.

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Purified human neutrophils from one buffy coat were re suspended in 5 ml of 0.4% (v/v) Perchloric acid (Sigma), sonicated for 1 min, and placed on ice for 30 minutes. The precipitated proteins and cell debris were removed by centrifuging at 3660 rpm for 30 mins, at room temperature. The perchloric acid was removed by titration with 0.4 M Potassium hydroxide (BDH), the reaction being monitored by a pH meter. The sample was placed on ice for a further 30 minutes, before the insoluble potassium perchlorate was removed by centrifugation at 3660 rpm for 20 mins. The sample was then prepared for reversed-phase HPLC analysis by lowering the pH to 3, using 1M Hydrochloric acid, and filtered through a 0.22 μ m filter.

2.13.) Oxidation of Methionine residue of Thymosin β 4

The Thymosin β 4 sample purified from neutrophils from a buffy coat, section 2.12.2.3., was divided into two equal parts, and both dried using a vacuum centrifuge, to approximately 20 μ l. The sample to be oxidised using the technique of Dr. Pappin, (ICRF, London, personal communication), was mixed with an equal volume of Hydrogen peroxide (100 volumes) (Sigma), and left for 5 minutes at room temperature. This sample, and the unoxidised sample, were then made up to 1 ml in 0.14% TFA (Buffer A) and applied to the reversed-phase chromatography column for separation of oxidised and non-oxidised thymosin β 4.

2.14.) Statistical analysis

Student's *t*-test was used to carry out statistical analysis of results.

RESULTS

3.1.) Development of the automated cell tracking assay data analysis

Chettibi et al., (1993) using the tracking assay method developed by Dow et al., (1987) showed that the supernatant from monocytes incubated in the presence of dexamethasone stimulated the locomotion of human neutrophils on albumin-coated glass. In contrast, supernatants from untreated monocytes contained factors which possessed the properties of known cytokines such as IL-8, which increased neutrophil adhesion and spreading, but also potentiated motility (Bignold et al., 1992; Kupper et al., 1992). Although the tracks of STMS-treated neutrophils were typically extended in comparison with IL-8 controls, of the three assay parameters described by Gail and Boone (1970, 1972) in their classic analysis of persistent random locomotion, namely speed, persistence and augmented diffusion, only speed was significantly enhanced by the STMS factor. This suggested a deficiency in the computation which failed to demonstrate the dispersive character of locomotion. It was therefore undertaken to extensively modify the existing cell tracking assay to investigate the contribution of speed and persistence to the dispersive character of STMS-induced motion and to obtain qualitative and quantitative data to characterise and assay the unknown factor.

3.1.1.) Modified cell tracking program

A modified cell tracking program was written in C by P.D. Lawrence and is as described in section 2.7.2.

This program produced files saved at the end of a tracking run that consisted of data containing the following information about the cells per

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five second interval: the number of pixels moved by each cell, the area of each cell, and the number of cells being measured.

In order to process this data to provide measurements of speed, diffusion coefficient, and persistence for the cell population being measured, a procedure was written in C language, and incorporated into the cell tracking program (Appendix I).

This procedure took the distance travelled by each cell in a five second interval, in pixels and converted it into distance in μm . This was done by calibrating the number of pixels on the screen to a micrometer on the microscope. This allowed calculation of the number of pixels per micron, and hence, the number of microns travelled by each cell per measured interval.

Plots were then made of mean displacement vs. time, and mean displacement squared vs. time. In order to extrapolate the gradient of the linear part of the displacement squared vs. time plot and the intersection of the line drawn at this point with the time axis a procedure was written that allowed the user, using the mouse control, to point on the computer screen to the start of this line and draw a line. The gradient of the line was calculated and the point of crossing the time axis displayed. This provided a convenient way of extrapolating from the plots the diffusion coefficient (gradient) and persistence (the intersection with the x-axis) (Fig. 11).

3.1.2.) Speed

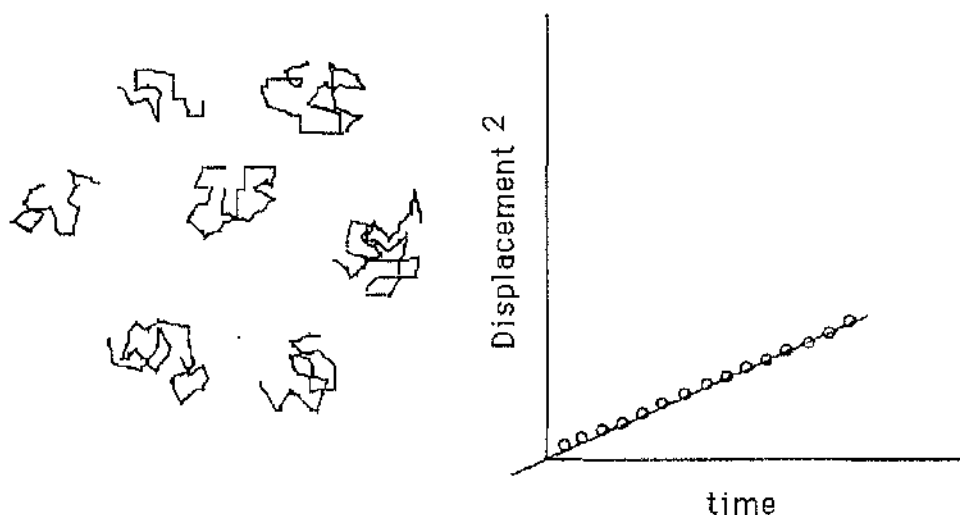
The displacement of randomly moving objects determined by the least squares approximation in a measured time interval gives an apparent speed that approaches the true speed as the measuring interval decreases.

Fig. 11. Diagram of theoretical cell motility parameters as described by Gail and Boone, 1970, 1972.

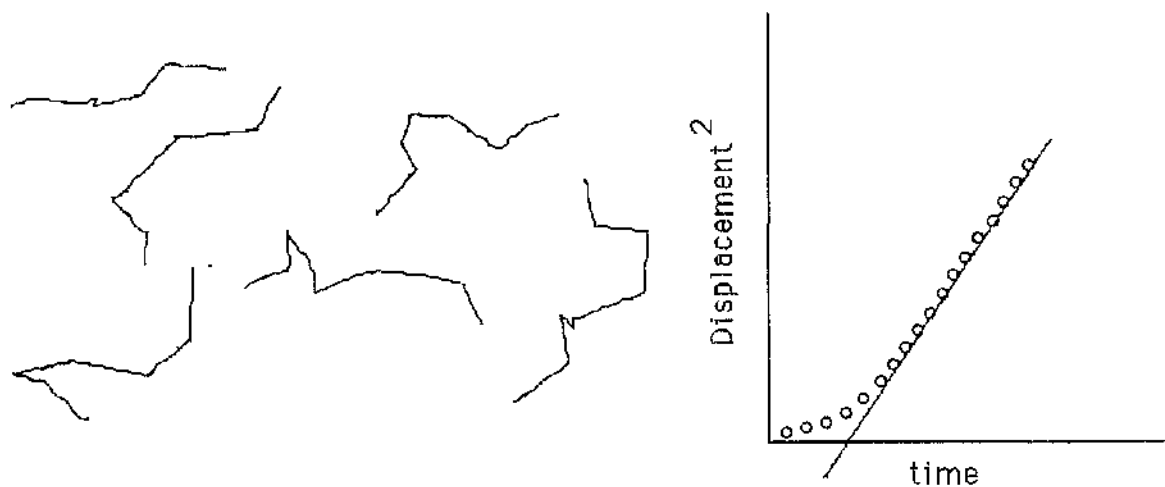
Augmented Diffusion Coefficient and persistence.

Diffusion Plots: Plot of the square of displacement as a function of time

Predict a linear diffusion plot for a true random walk



Predict a biphasic plot for persistent random locomotion



Slope of the linear portion gives the diffusion coefficient and the point at which the extrapolation of this line cuts the time axis has been identified as the persistence of locomotion

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However the use of short time intervals reduces the signal to noise ratio and a compromise must be sought. In the present case a 5-second time interval was chosen because it allowed the assay progress to be assessed by eye. It was clear however that the use of a 5-second interval gave considerably higher cell speeds than did a 10-second interval (result not shown), hence even the 5-second interval must underestimate the true speed. Nevertheless for the results displayed in table 1, the average distance covered in 5 seconds was very similar for each of the stimuli used. Speed was not therefore a sensitive parameter for distinguishing different stimuli or for analysing the concentration dependence of a single stimulus. It was also of interest that control cells, which were clearly non-motile, returned significant speed values as shown in table 1. This apparent speed of non-motile cells could be explained by pixel jitter, altering the apparent centre of the cells, and convection currents in the chambers as the temperature equilibrates.

The average speed over the complete measurement time was expressed in $\mu\text{m}/\text{min}$, and used as an indicator of cell motor activation.

3.1.3.) Drift

The non-random component of movement of the whole cell population should be zero in the absence of a chemotactic gradient, but for small sample sizes where short-term displacement cannot be symmetrical, or for fields where other conditions are non-uniform, a component of non-random locomotion, called here drift, is always present. Where a non-random trend persists over long time periods it could, in principle, mask the effects of random or pseudo-random locomotion. Non-random movement was calculated by determining the centre of cell density by

Table 1. The diffusion coefficient, persistence and speed of locomotion of human neutrophils treated with various stimuli

Treatment	Diffusion coefficient ($\mu\text{m}^2 / \text{sec}$) \pm s.e.m.	Persistence (secs)	Speed(+ drift) ($\mu\text{m} / \text{min}$) \pm s.e.m.	Speed (-drift) ($\mu\text{m} / \text{min}$) \pm s.e.m.
BSS	0.007 \pm 0.001	0	4.9 \pm 0.3	2.0 \pm 0.3
ppSTMS	0.77 \pm 0.09	37.2 \pm 4.3	14.6 \pm 0.1	11.2 \pm 0.2
STMS	0.784 \pm 0.09	43.4 \pm 9.8	13.9 \pm 0.9	11.7 \pm 0.5
CMS	0.05 \pm 0.02	5.0 \pm 1.5	8.0 \pm 0.8	5.0 \pm 0.8
fMLP	0.14 \pm 0.02	3.5 \pm 0.7	15.3 \pm 1.1	11.3 \pm 1.0
IL-8	0.12 \pm 0.03	1.8 \pm 0.5	15.2 \pm 0.6	10.7 \pm 0.6
LPA	0.09 \pm 0.02	1.9 \pm 0.7	15.0 \pm 0.8	11.4 \pm 0.9
CM	0.03 \pm 0.008	0	7.9 \pm 0.58	5.1 \pm 0.6

Adherent human neutrophils were treated with 400 μl of balanced salt solution (BSS), partially purified steroid-treated monocyte supernatant (ppSTMS); control monocyte supernatant (CMS); 10^{-8} M formyl-methionyl-leucyl-phenylalanine (fMLP); 100 ng/ml interleukin-8 (IL-8); 20 μM lysophosphatidic acid (LPA); and culture medium (CM).

The persistence value of zero for BSS and CM is a product of the low gradient of the displacement squared vs. time plot making it impossible to extrapolate the persistence value, so the value is assumed to be zero. The results represent the mean \pm s.e.m. of at least five experiments.

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carrying out a vectorial sum of the X and Y displacements of each cell centre to determine the average population displacement. This was used to calculate drift per frame and thereby the component of speed that could be attributed to drift alone. However because target cell loss can reduce cell numbers to the point where lack of symmetry produces spurious drift, the programme was constructed to display the results corrected for the non-random component of locomotion, but also displaying the non-corrected locomotion. Although there was considerable drift per frame, the long-term drift of control cells was negligible. This suggested that the short-term effects were self-cancelling and time-lapse video playback showed a very clear gyratory locomotion that might underlie such a short-term drift phenomenon. When the speed of control cells was corrected for drift per frame, it was reduced to more apparently realistic levels (table 1). Of the various locomotor stimulants tested, STMS tended to give rise to large drift components, although these were variable in magnitude. Many of the responses to CMS and IL-8 also appeared to contain a large drift component, but this always occurred when the number of active target cells fell below around 20. These stimulants tended to induce reversible phase-darkening with consequent target-cell loss and the apparent drift could be attributed to lack of population symmetry which is an inevitable consequence of small sample size.

In order to monitor the drift component of the displacement a procedure was written that calculated the average drift of the cell population, and direction of drift over the measured time. This allowed the user to note any consistency in the direction of drift, perhaps indicating a slope of the slide, or microscope stage.

3.1.4.) Cell displacement

As the first step in calculating diffusion parameters, the mean accumulated displacement of cells from their original centres was plotted as a function of time, both with drift included and subtracted. The plots vary from moderately linear for STMS to parabolic for fMLP (fig. 12). A linear plot would indicate that the cells were maintaining their initial direction of motion throughout the measuring period. The parabolic curve indicates that the cells are changing direction frequently throughout.

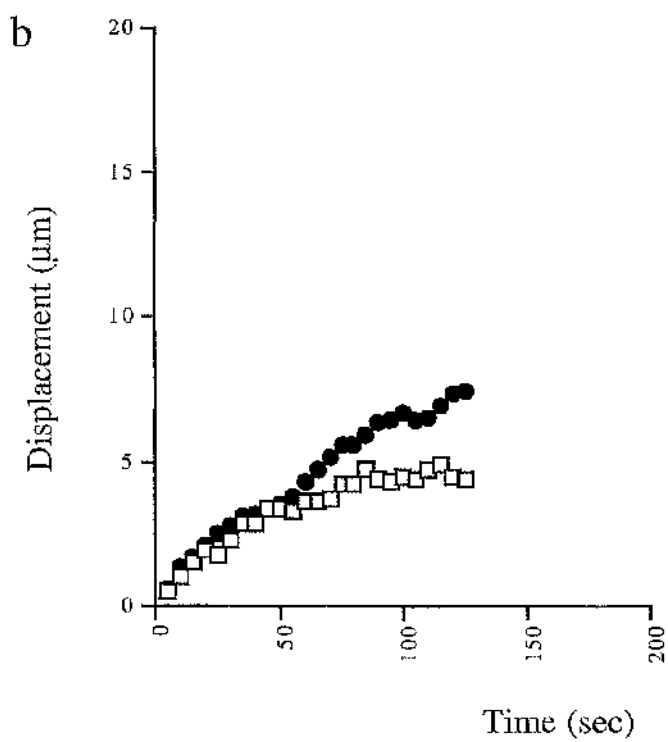
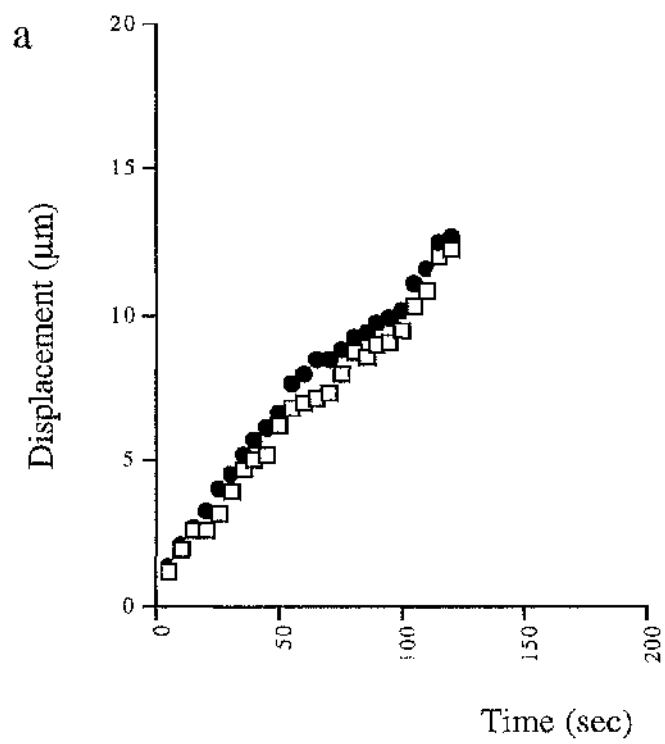
3.1.5.) Cell-track display

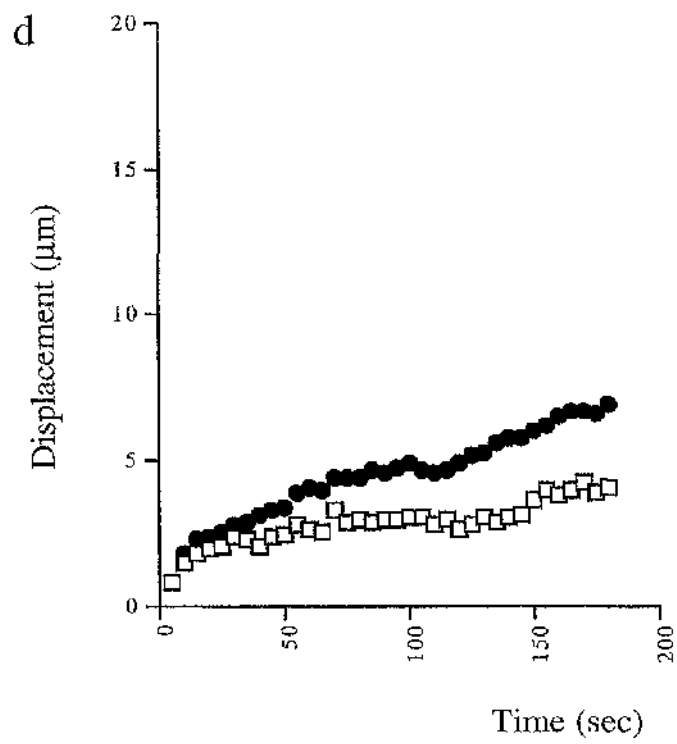
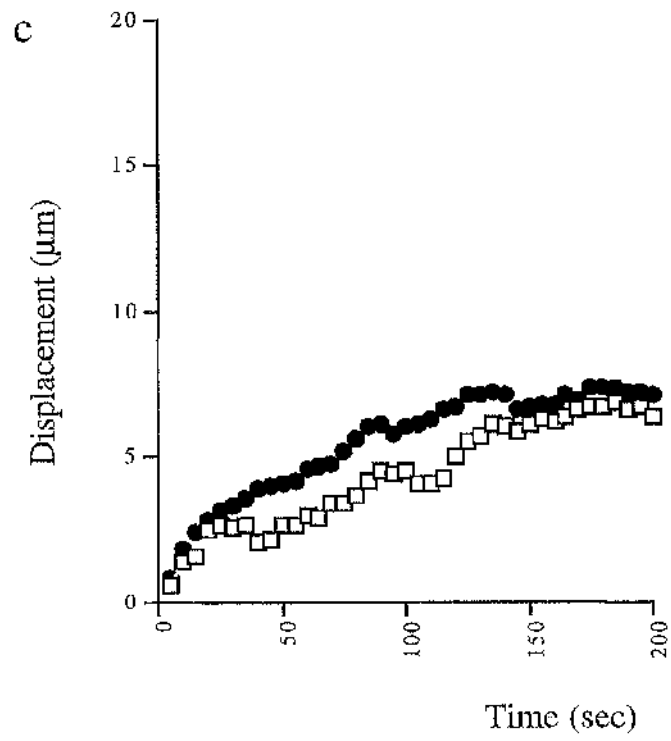
In order to display the dispersive character of neutrophil cell populations being assayed, a normalisation procedure was adopted in which the starting co-ordinates of each target cell were translated to the origin of an x-y plot at the screen centre, and the movement of each individual cell represented by a line connecting its centre position at each interval. Comparison of the linear tracks produced showed greater elongation when stimulated by STMS than by CMS or control culture medium in the presence or absence of 10^{-6}M dexamethasone (CM) (Chettibi et al., 1993). This method, applied to compare tracks obtained in response to CM, STMS, CMS, fMLP, IL-8 and LPA clearly demonstrated that the dispersive power of STMS greatly exceeded that of all the other agents tested (Fig. 13). The surprising result produced with LPA led to investigation of the action of LPA on other neutrophil functions (section 3.12.)

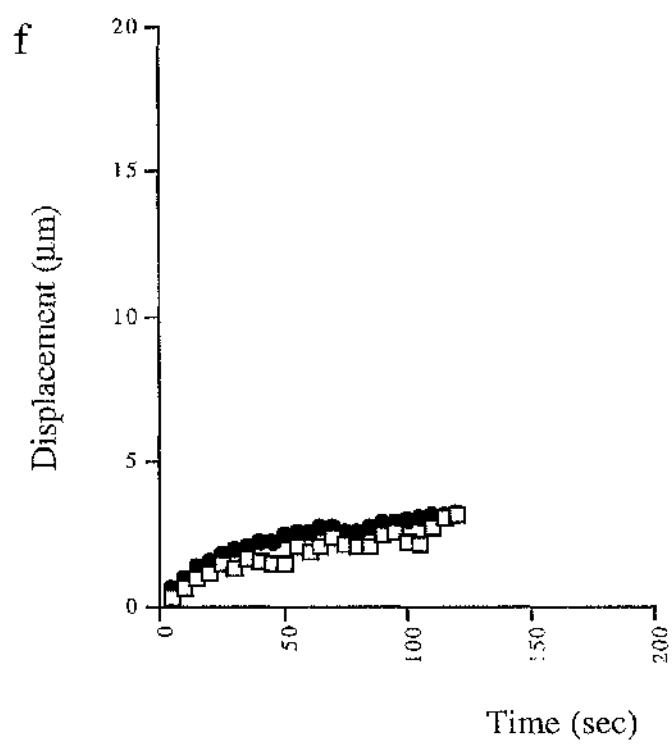
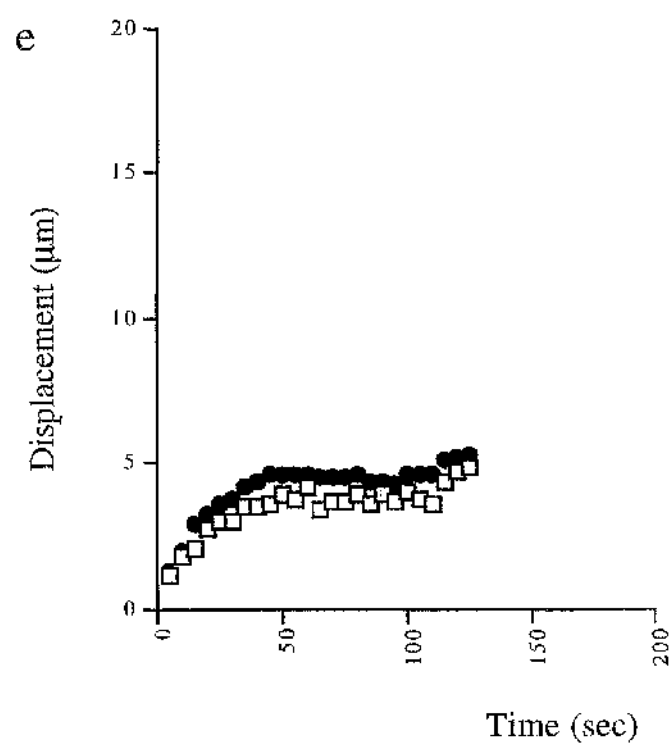
Fig. 12. Plots of the mean accumulated displacement of neutrophils in response to various stimuli.

Adherent human neutrophils were treated with 400 μ l of: (a) Steroid-treated monocyte supernatant (STMS); (b) Control monocyte supernatant (CMS); (c) 10^{-8} M *n*-formyl-methionyl-leucyl-phenylalanine (fMLP); (d) 100 ng ml $^{-1}$ interleukin-8 (IL-8); (e) 20 μ M lysophosphatidic acid (LPA); and (f) Ham's F-10 culture medium (CM).

Uncorrected displacement (●), drift-corrected displacement (□).







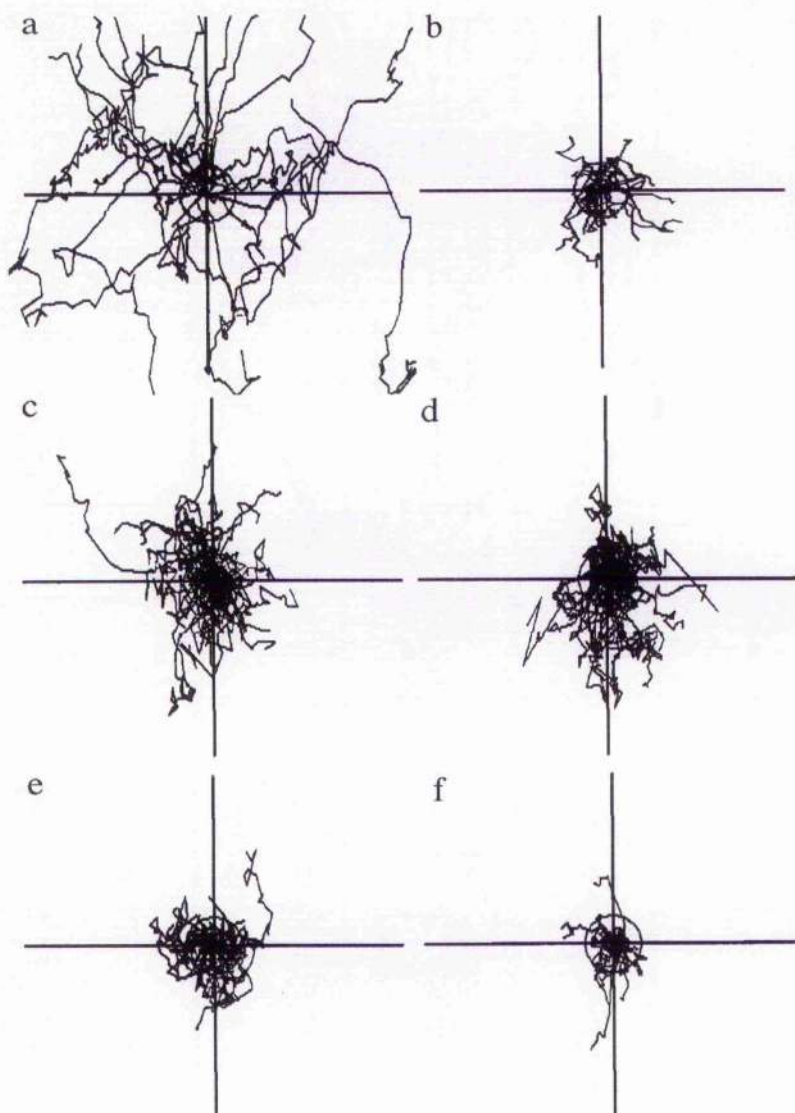


Fig. 13. Comparison of tracks obtained in response to various neutrophil stimuli.

The starting position of each cell was normalised to the origin of the axes. The circle at the origin represents a cell of 10 μm diameter. Tracks were obtained over an assay time of approximately two minutes. Adherent neutrophils were treated with approximately 400 μl of (a). steroid-treated monocyte supernatant (STMS) (b). control monocyte supernatant (CMS). (c). 10^{-8} M formyl-methionyl-leucyl-phenylalanine (fMLP). (d). 100 ng/ml interleukin 8 (IL-8). (e). 20 μM lysophosphatidic acid (LPA). and serum-free Ham's F-10 culture medium (CM).

3.1.6.) Augmented diffusion

Analysis of cell dispersion by the random walk method as described by Gail and Boone (1970, 1972) was based on the plot of the square of displacement as a function of time which, for convenience, is called here a "diffusion plot". Theoretical models predict a linear diffusion plot for a true random walk and a biphasic plot for persistent random locomotion in which an initial rising curve becomes linear. In both cases the slope of the linear portion gives the diffusion coefficient and the point at which the extrapolation of this line cuts the time axis has been identified as the persistence of locomotion. They also showed that persistence increases the rate of population dispersion and augments the diffusion coefficient by the factor $(1+p)$ where p is the persistence time. Their treatment only considered the limiting cases where $p = 0$ or $p < t$, where t is the elapsed time, but they showed that extrapolation of the linear portion of the diffusion plot to the time axis gives the persistence time. Thus for random locomotion the diffusion coefficient $D = S^2$, where S is the instantaneous speed of locomotion. For persistent random locomotion the augmented diffusion coefficient $D^* = S^2 (1+p)$ where $p < t$.

Figure 14 demonstrates that only STMS-treated cells showed a non-linear plot of this type which could be interpreted as persistent random locomotion. After STMS, the next most potent dispersing agent IL-8 showed the characteristics of an agent that stimulated non-persistent random locomotion. In contrast, movement stimulated by fMLP showed the unexpected quality of constrained diffusion where the diffusion plot falls below the extrapolated initial linear portion. This indicates that the cells can make large initial excursions from their original positions but are tethered by means of strong adhesions and have a low probability of productive locomotion. Because drift is, in principle, time-independent

and therefore cumulative, it is quite clear that a high drift component will give rise to a hyperbolic diffusion plot. For this reason all of the diffusion curves were plotted both with drift present and with drift removed. The results demonstrate that high drift makes a major contribution to the apparent persistence and illustrate that careful analysis of the drift component is a prerequisite for interpreting tracking data in terms of persistent random locomotion. Nevertheless it is clear that drift is not the major contributor to persistence in the majority of STMS assays (Fig. 14).

3.2.) Assays for STMS activity

STMS is obtained as a culture supernatant based on Ham's F-10 medium which itself has a stimulatory effect on neutrophil migration speed and a minor effect on diffusion coefficients. For this reason all of the comparisons given in this study are of locomotor stimulants also presented in Ham's F-10 medium. Table 1 shows the migration parameters for the various stimulators. The results illustrated that the tracking assays can be used for quantitative estimation of any of the pure stimulating agents, giving migration speeds in the range 5-20 μm per minute. However, the diffusion coefficient is a more sensitive parameter varying over the range 0.03-1 μm^2 per second with the lower limit strongly dependent on the nature of the medium. Because STMS alone gives the higher values, it follows that the diffusion coefficient provides a large measure of discrimination between this and other activities. Dose response curves which show the relationship between speed, persistence and augmented diffusion for STMS illustrate that despite the large range of diffusion coefficients obtained, the errors in this bioassay are also

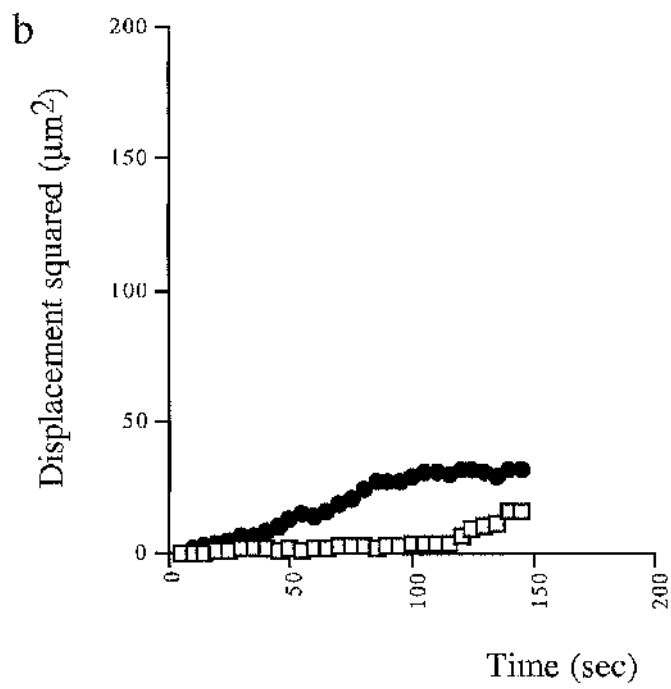
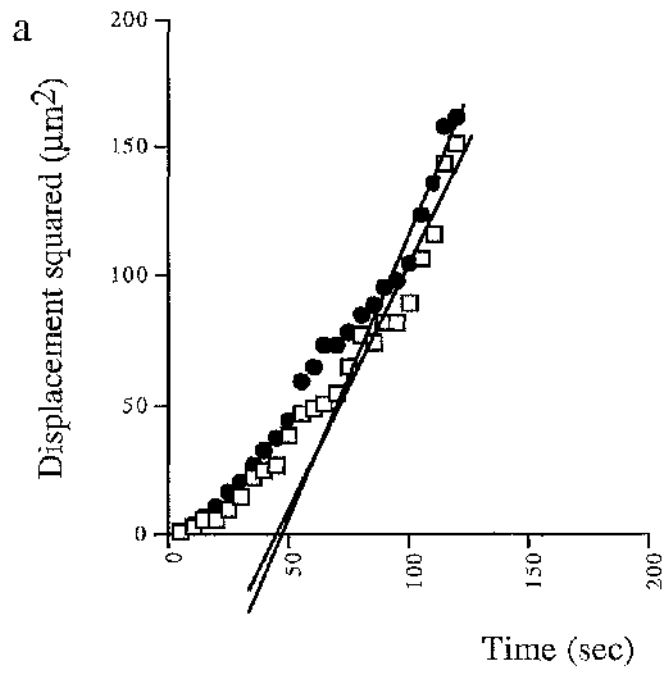
Fig. 14. Plots of the square of the mean accumulated displacement squared vs. time of neutrophils in response to various stimuli.

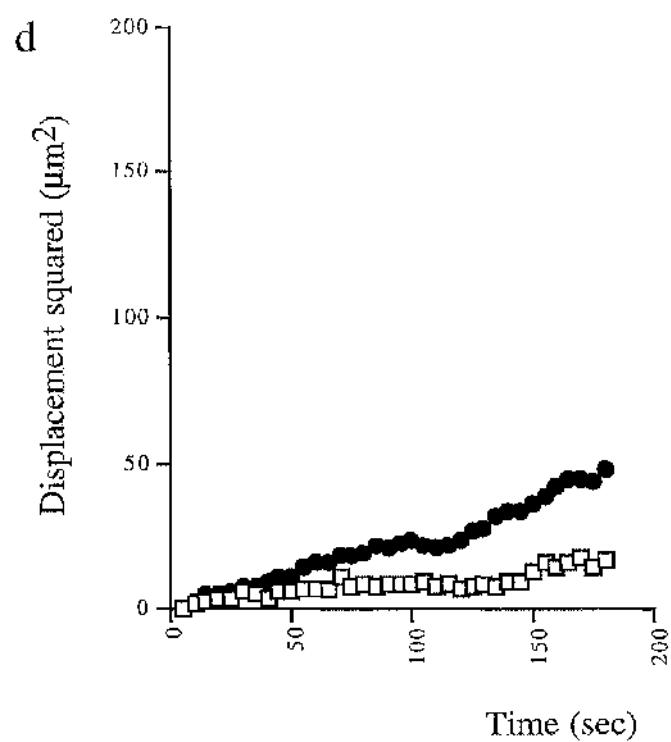
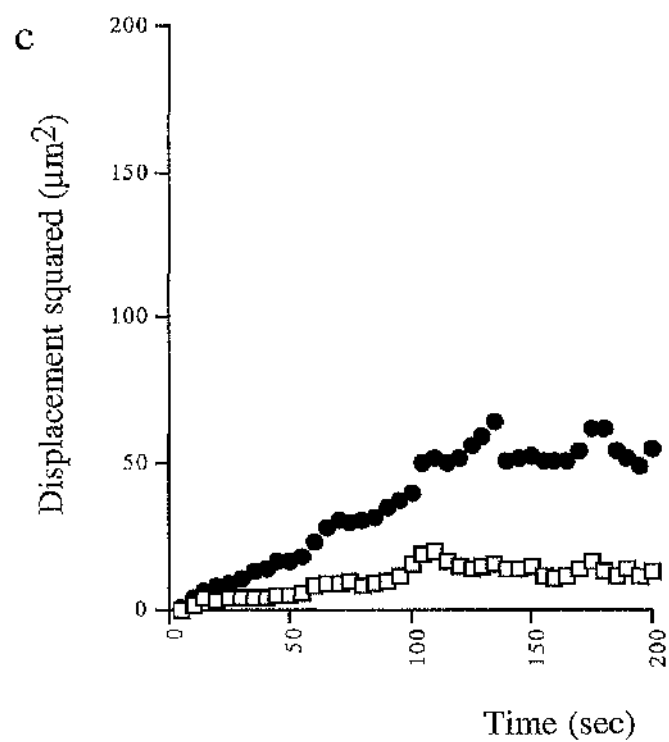
The data from fig. 12. were replotted as the square of accumulated displacement as a function of time and used to determine the augmented diffusion coefficients and the persistence times.

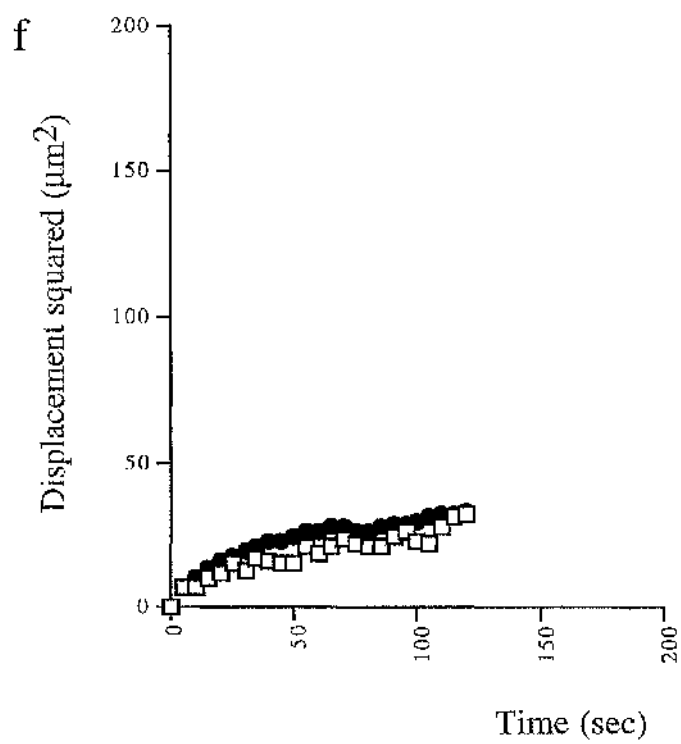
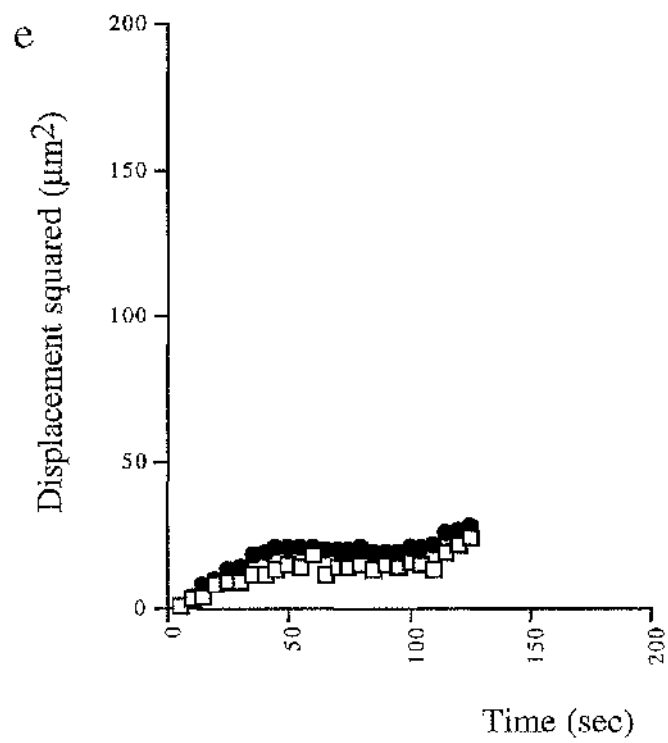
(a) Steroid-treated monocyte supernatant (STMS); (b) Control monocyte supernatant (CMS); (c) 10^{-8} M *n*-formyl-methionyl-leucyl-phenylalanine (fMLP); (d) 100 ng ml⁻¹ interleukin-8 (IL-8); (e) 20 μ M lysophosphatidic acid (LPA); and (f) Ham's F-10 culture medium (CM).

Uncorrected data (●), drift-corrected data (□).

Straight lines drawn through the linear part of the uncorrected curves and the drift corrected curve for STMS gave persistence values of 48 and 47 seconds, respectively.







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large and thus limit the precision. Thus the tracking assay provides a reasonably quantitative assay of the factor and is relatively insensitive to possible contaminating activities.

During the purification of STMS different assay conditions are obtained because the sample is no longer present in Ham's F-10 culture medium and it is clearly advantageous to use an assay medium that has a minimum effect of neutrophil locomotion. The two choices available are balanced salt solution (BSS) or Hank's HEPES. Both media are suitable for the assay of STMS and have the great advantage that they do not stimulate the locomotion of control cells. Table 1 shows the results when BSS was used, however, Hank's HEPES was used in later purification procedures due to ease of preparation and storage.

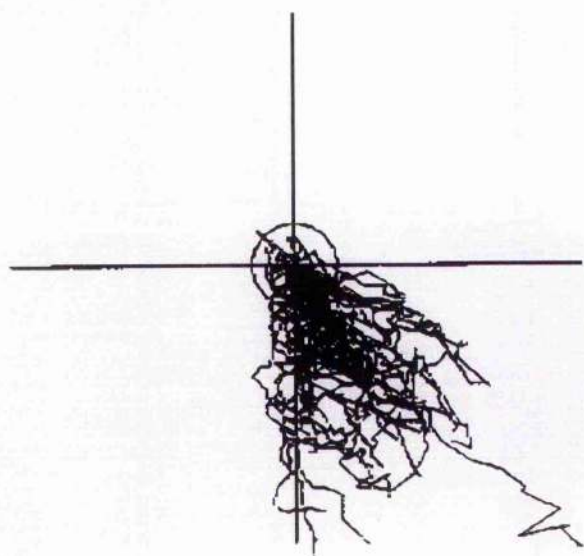
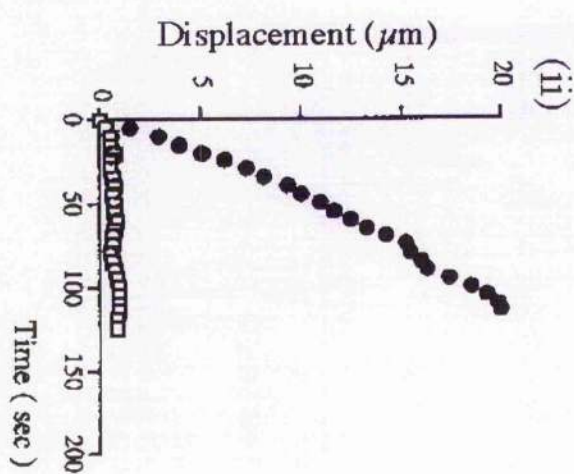
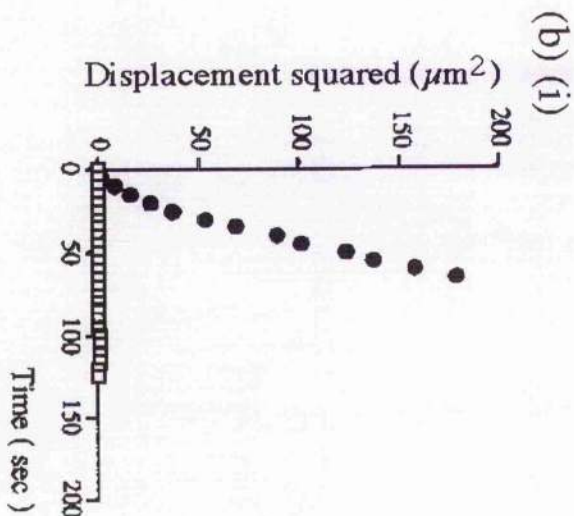
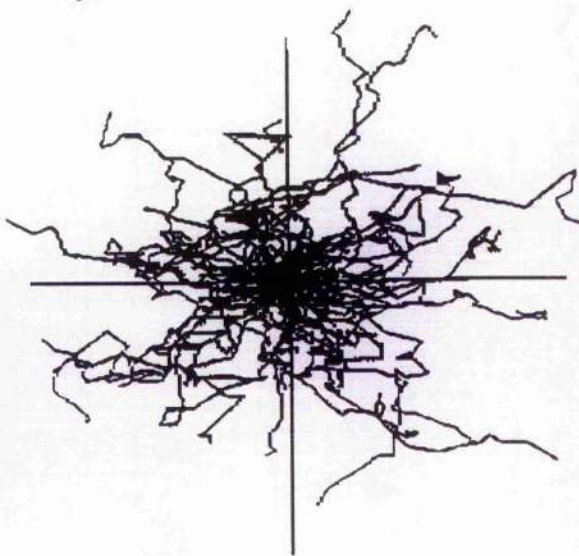
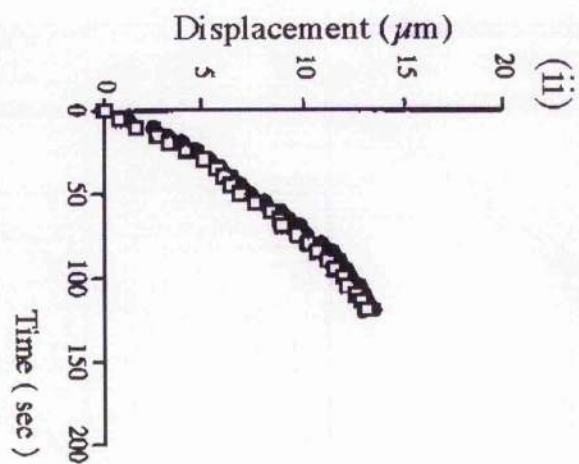
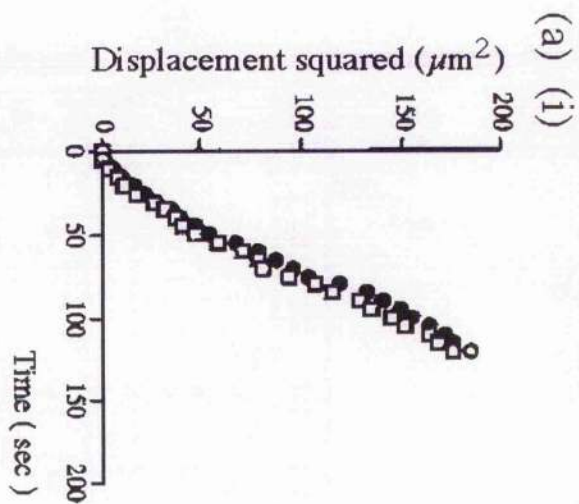
Figure 15 shows that vacuum dialysis of STMS culture medium followed by dilution in BSS gives a preparation which remains highly active both in the dispersion assay and in the tilted-stage assay.

3.3.) Tilted-stage experiments

Neutrophils moving in response to STMS showed a relatively large drift component of locomotion even when cell numbers were sufficiently high to ensure a symmetrical population distribution, indicating that the residual drift must be due to inherent asymmetry in the system. Because STMS was known to lower the adhesion of these cells to the surface it could increase their sensitivity to convection currents or to a non-level stage. It was therefore of considerable practical and theoretical interest to see how the cells responded to a gravitational field and this was achieved by tilting the whole microscope laterally by 30° . After a brief settling period, cells treated with any of the stimulating agents resumed steady

Fig. 15. The effect of vacuum dialysis on the activity of STMS.

- (a) Plot of the square of displacement as a function of time on level surface
(b) Plot of the square of displacement as a function of time on a 30° tilted surface. (i) Plot of displacement squared as a function of time. Square of uncorrected displacement (\bullet), square of drift-corrected displacement (\square)(ii) Plot of mean accumulated displacement as a function of time. Uncorrected displacement (\bullet); drift-corrected displacement (\square). (iii). cell tracks normalised to the origin of the axes.

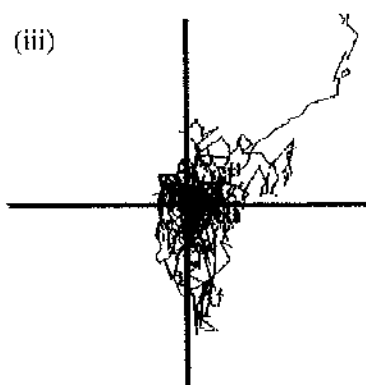
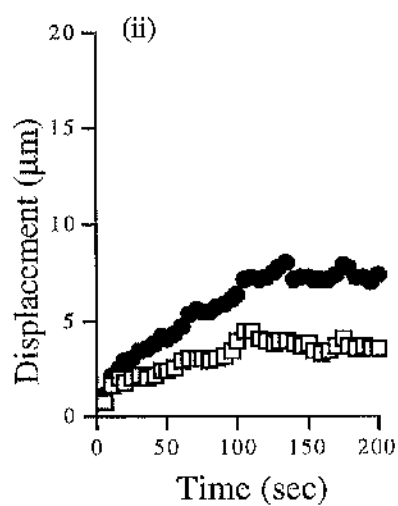
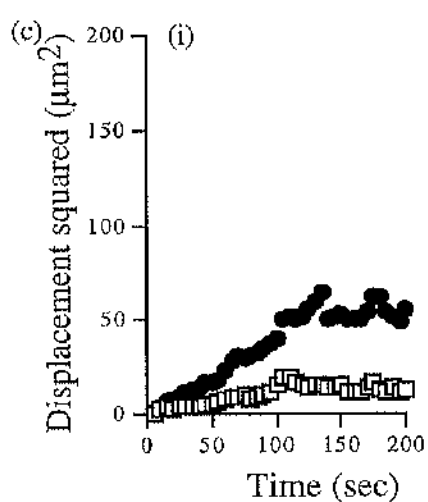
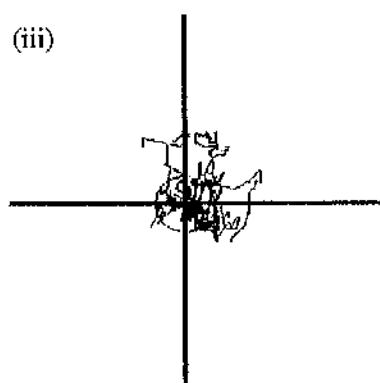
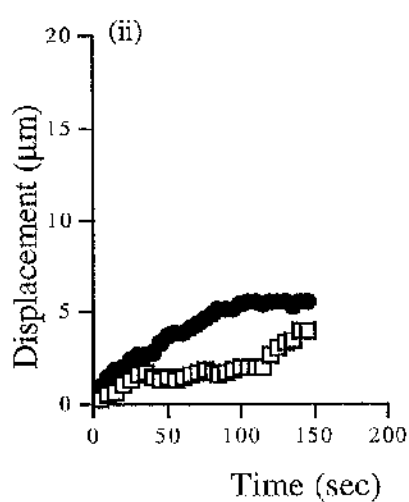
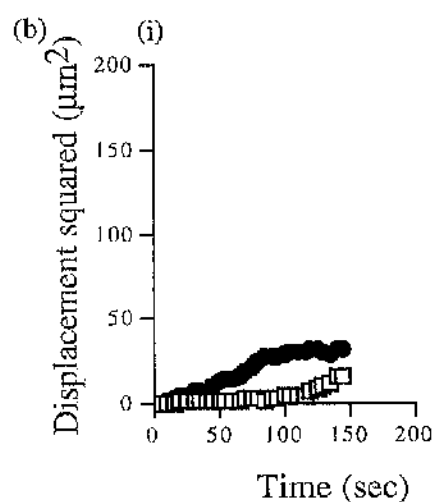
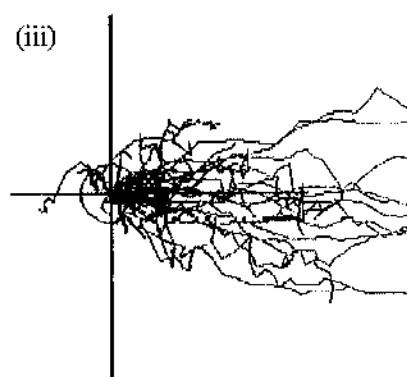
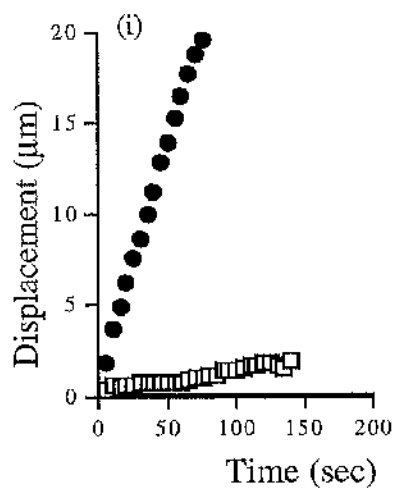
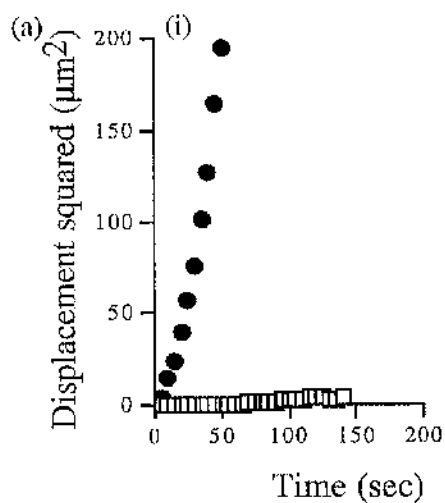


Results

activity. All cells, even the unstimulated controls, showed some tendency to move down-slope, but the behaviour of STMS-treated cells was most dramatic (Fig. 16). Using the analysis program with automatic drift correction, all other stimulants induced random (radial) locomotion with an increased drift component. For STMS-treated cells, in contrast, the radial component virtually disappeared and movement was dominated by non-random movement down-slope. It was highly significant however, that although the direction of locomotion was strongly polarised, the rate of locomotion was increased by no more than 50%. Such an effect could be attributed to the reduction in the random component of locomotion because increased track linearity could, in principle, cause a significant increase in the apparent speed measured over a 5 second time interval. Unattached cells clearly exceeded the maximum tracking speed of around $1\mu\text{m}$ per second, but the rate of target cell loss although increased in tilt experiments, was not dramatic and cells that remained as tracking targets were clearly seen to be moving from one position of adhesion to another. Because the locomotion of cells responding to STMS is highly directional, the appropriate analysis is of displacement rather than the square of displacement. Clearly the drift correction is no longer appropriate. The displacement curves show the characteristic high value for the first 1-2 giving way to a moderately linear curve typical of tethered random locomotion. Comparison of the slope of the sustained linear part of the curves give values with greater discrimination between the effects of STMS and CMS/fMLP (2.5 and $0.47 / 0.48\mu\text{m}$ per second respectively) than obtained in the radial dispersion assay (table 1) with a higher degree of confidence in the individual values.

Fig. 16. The effect of a gravitational field on neutrophil tracking

Adherent human neutrophils were treated with 400 μ l of: (a). steroid-treated monocyte supernatant (STMS); (b). control monocyte supernatant (CMS); and (c). 10⁻⁸M formyl-methionyl-leucyl-phenylalanine (fMLP). The assay chamber was then tilted by 30 $^{\circ}$ and measurements commenced after a delay of 1 minute. (i) Plot of displacement squared as a function of time. Square of uncorrected displacement (\bullet), square of drift-corrected displacement (\square); (ii) plot of the accumulated mean displacement as a function of time. Uncorrected displacement (\bullet), drift-corrected displacement (\square); (iii) cell tracks normalised to the origin of the axes.



A caveat to this assay system, that must be pointed out at this stage, is the inconsistency in the behaviour of human neutrophils from different individuals in our tracking assay. As will be demonstrated later, under apparently very similar circumstances, neutrophils from different individuals can have very different basal activity which affects the tracking parameters.

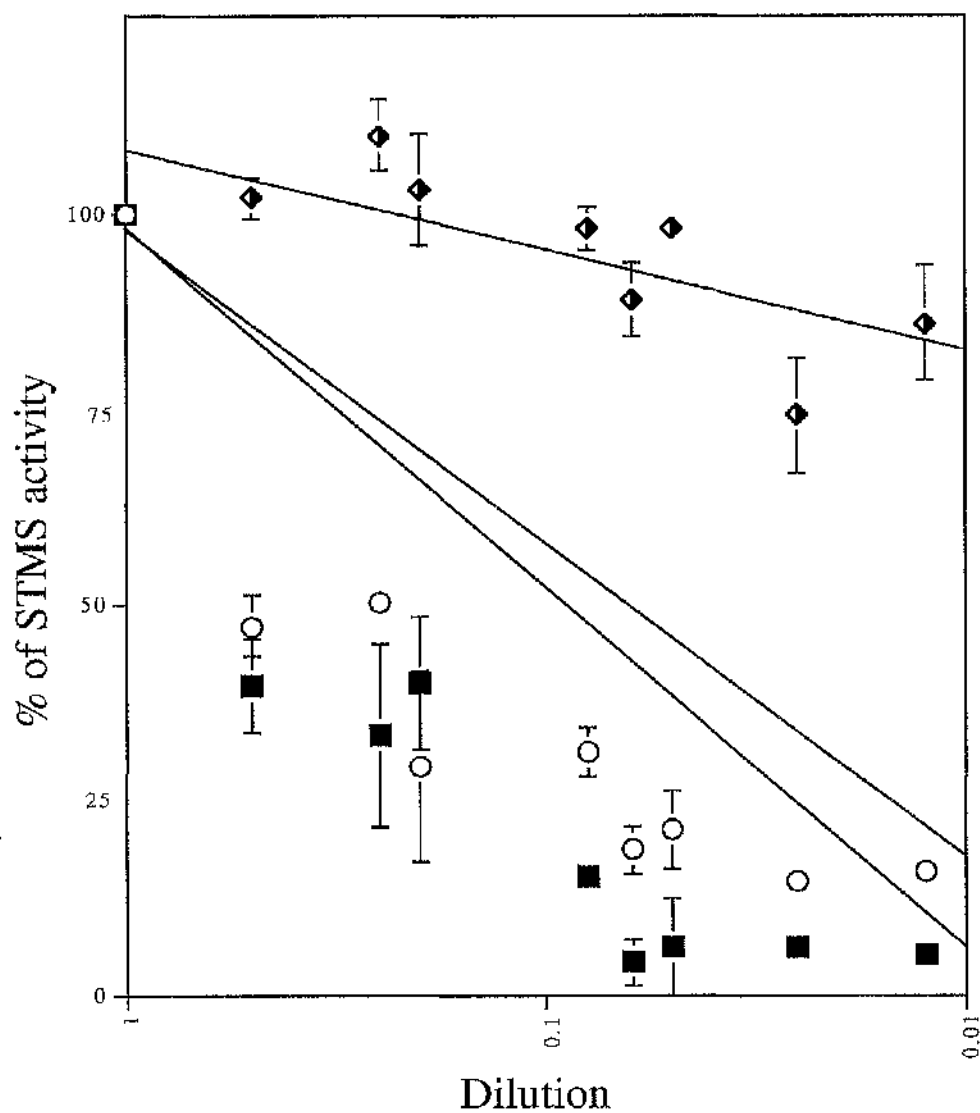
Blood for neutrophil tracking is normally provided for us by volunteer medical students from The Glasgow Royal Infirmary, and is taken under similar circumstances each day. However, the variation in neutrophil behaviour can be very significant. On some days the cells undergo homotypic aggregation to such an extent that assays are impossible. Other days the cells stick to the BSA-coated coverslips so strongly that tracking becomes impossible due to too many cells per field, whilst other times very few cells stick to the coverslip at all. There are also problems with basal activity at both extremes. i.e. some samples will have cells that will not respond to any stimuli, whilst other cells are activated and motile without any stimulation.

3.4.) Dose response of starting material

It was of great interest for purification of this factor to obtain a dose response curve of the activity being measured in STMS. This would allow us to optimise the conditions of assay during the purification procedure, as we would have both highly concentrated and dilute samples during different stages. A dose response curve was produced by diluting starting material STMS in Ham's F-10 serum-free culture medium (Fig. 17).

There was a rapid reduction in both the diffusion coefficient and persistence of the cell populations as the starting material was diluted to

Fig.17. The effect of different dilutions of STMS on the diffusion coefficient, persistence and the speed of locomotion of human neutrophils.



Diffusion coefficient (○), persistence (■) and speed (◆)

Results

1/8 of starting material, similar to the dose response of Stevenson (section 1.2.6.) but the reduction in the speed of locomotion was not so dramatic. This confirmed that speed was not a good parameter for our active factor, but the reduction in diffusion coefficient also indicated that the concentration used in assays was very important if the activity was to be detected after each purification step.

3.5.) Adhesion of human neutrophils to bovine aorta endothelial cells

The adhesion of neutrophils to the endothelium in post-capillary venules is a very important stage in the inflammatory process (Anderson and Springer, 1987), and is a potential target for anti-inflammatory therapy. Initial results of Chettibi et al., 1993, showed that STMS starting material decreased the adhesion of human neutrophils to albumin coated coverslips, when compared with CMS and Culture medium +/- steroid. It was of interest to see if these results could be repeated with a more physiologically relevant substrate for adhesion. Bovine aorta endothelial cells were used as this substrate. The results show that the adhesion of neutrophils to bovine aorta endothelial cells is indeed reduced when compared with the controls (Fig. 18).

3.6.) Effect of STMS on the fMLP induced chemotaxis of human neutrophils

Preliminary observations of neutrophil chemotaxis using the modified Boyden chamber revealed a striking contrast between the response to STMS and fMLP. Neutrophils showed massive invasion of the filter when

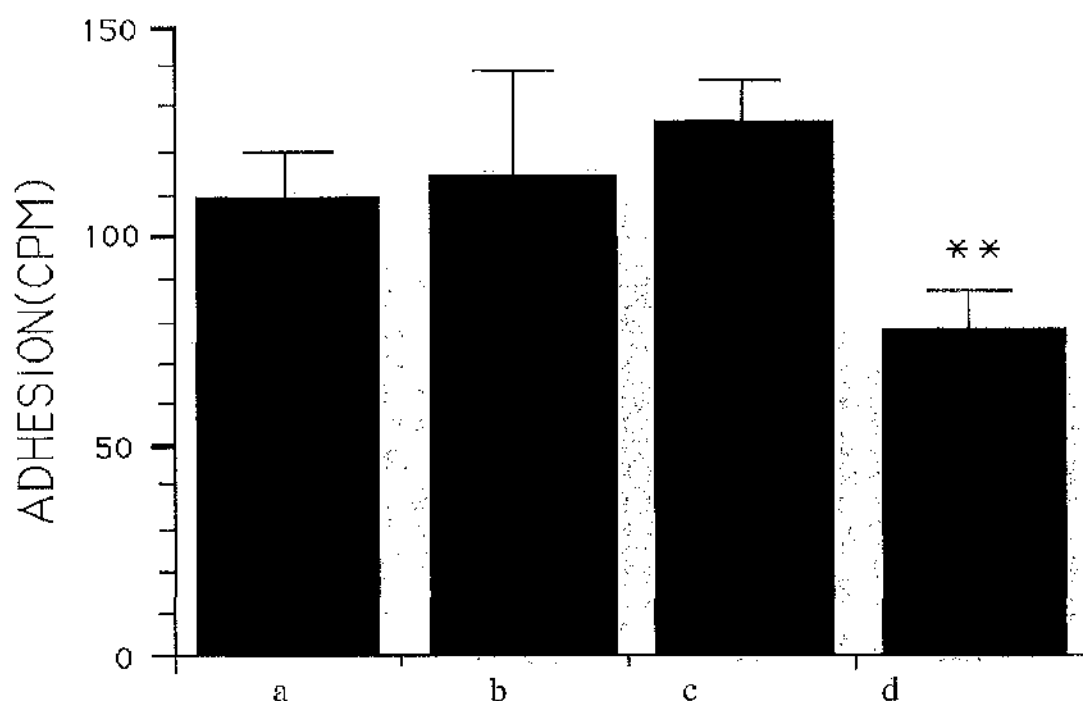


Fig. 18. Effect of supernatants from human monocytes cultured for 24 hours at 37°C, with and without 10^{-6} M dexamethasone, on the adhesion of human neutrophils to bovine aorta endothelial cell monolayers.

(a) culture medium without dexamethasone; (b) culture medium with 10^{-6} M dexamethasone; (c) Control monocyte supernatant (CMS); (d) Steroid-treated monocyte supernatant (STMS).

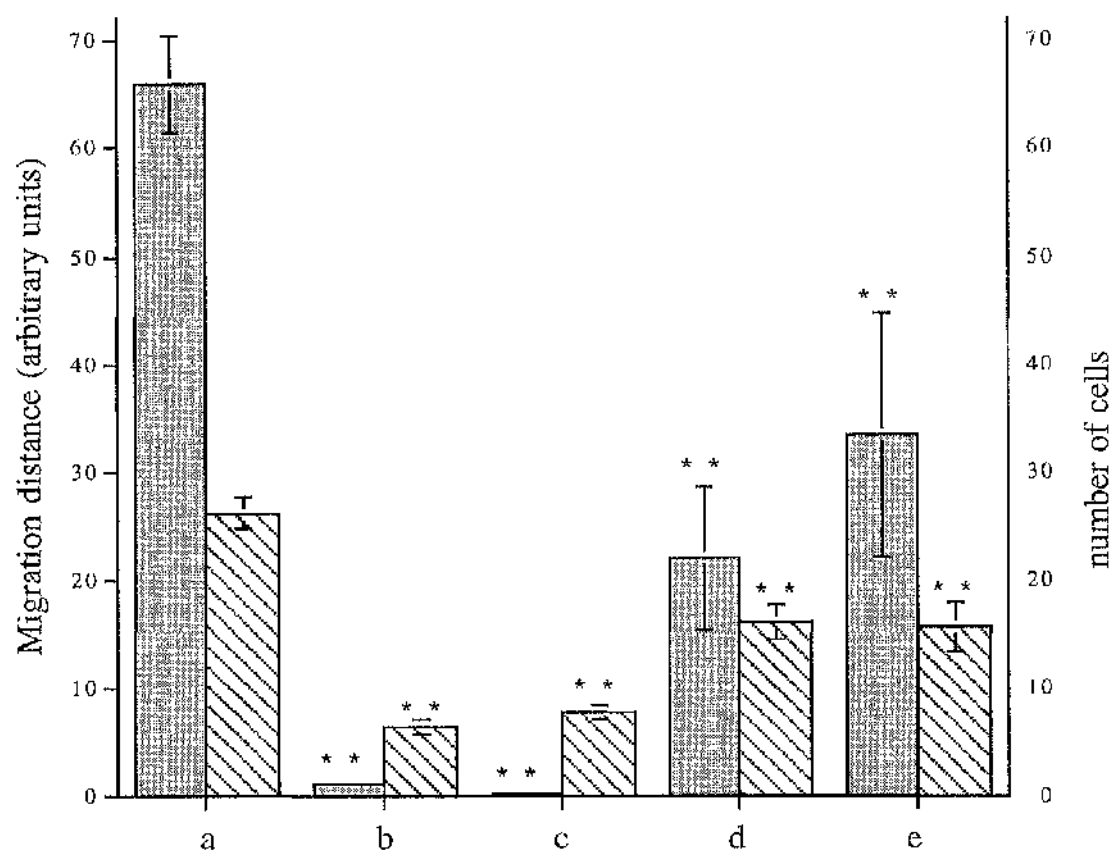
Mean \pm s.e.m. (vertical bars) $n = 3$. $P < 0.05$ given by **.

fMLP was present in the lower chamber, but gave no such response to STMS. However when the filters were examined by the leading front method it became clear that a few STMS-treated cells were able to invade successfully, but in low numbers, in keeping with the predictions for persistent locomotion. These observations suggested that a more suitable analysis would be to measure total invasion, or to determine the number of cells present midway between the leading front and the surface of the filter. Figure 19 presents the analysis of chemotactic assays using both the leading front and the average invasiveness methods. The results showed that STMS was not itself chemotactic, but when present in the upper chamber, dramatically inhibited the response to fMLP. In a uniform concentration of fMLP (i.e. present in both chambers) there was a similar reduction in invasiveness.

3.7.) The contribution to Dispersive locomotion of F-Actin

To investigate the underlying basis of the shape differences and to understand the nature of dispersive locomotion, the distribution of polymerised actin in the cells was examined. Control non-activated neutrophils showed a weak, rather punctate distribution of fluorescence, but with no sign of a major polarised focus corresponding to a relatively even distribution of cortical actin (Fig. 20 a). The results obtained for fMLP were similar to those presented by other workers (Coates et al., 1992) and are consistent with the interpretation that actin polymerisation is much more intense than in control cells and is associated with points of adhesive contact that are foci of active locomotion (Fig. 20 b). Cells that were highly spread in response to CMS or to $\text{TNF}\alpha$ showed an extremely punctate distribution of F-actin (Fig. 20 c,d). The pattern of actin staining

Fig. 19. The inhibition of fMLP-induced chemotaxis of human neutrophils by STMS measured using a modified Boyden chamber assay.



(a) upper chamber - culture medium with dexamethasone; lower chamber - 10^{-9} M fMLP; (b) upper chamber - STMS; lower chamber - 10^{-9} M fMLP; (c) upper chamber - 10^{-9} M fMLP; lower chamber - 10^{-9} M fMLP; (d) upper chamber - culture medium with dexamethasone; lower chamber - STMS (e) upper chamber - culture medium with dexamethasone; lower chamber - culture medium with dexamethasone. Number of cells which have migrated half of the mean migration distance of positive control(A). Cell-front migration distance.

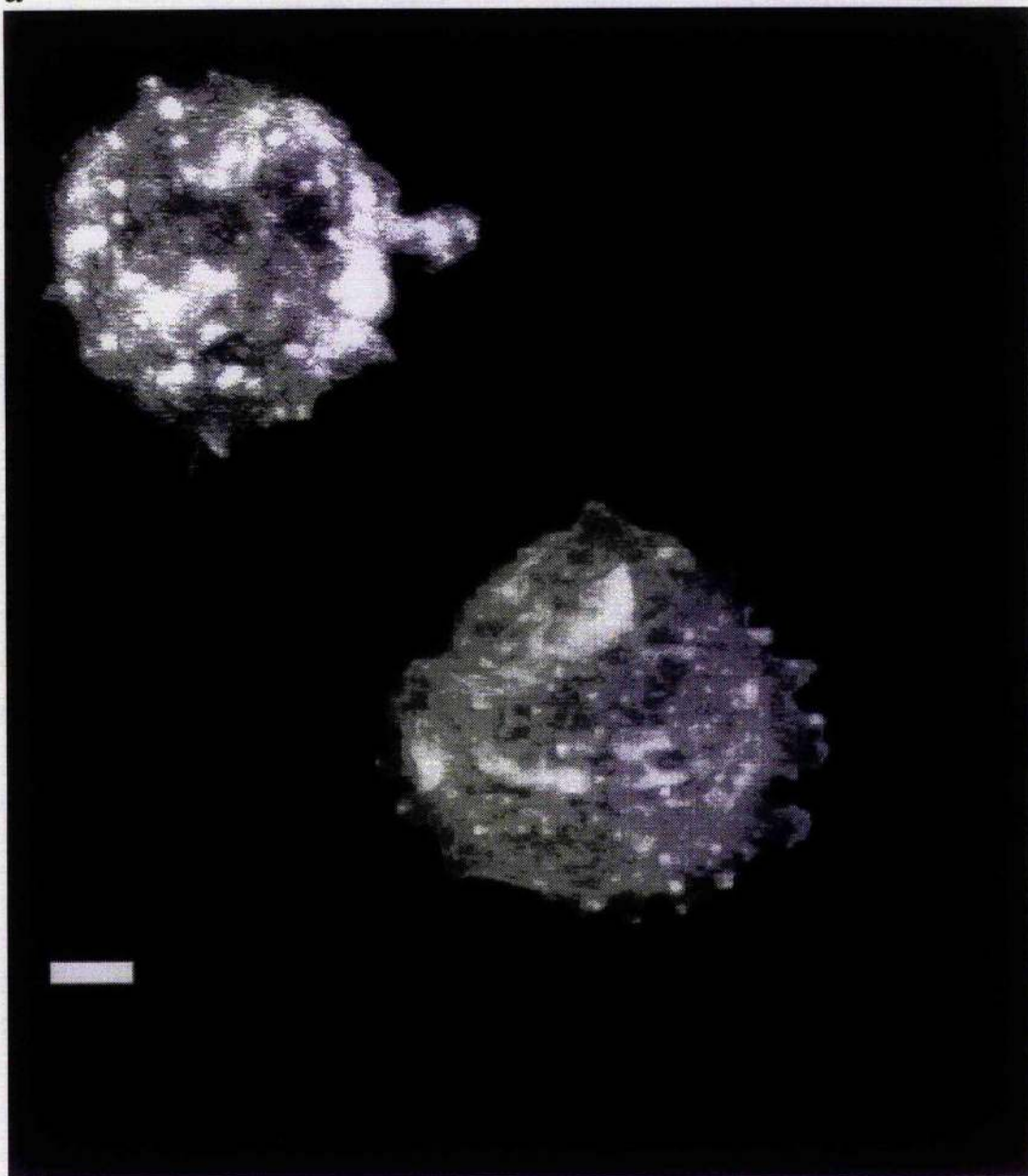
Five randomly selected fields were counted for each filter. Values shown are mean \pm s.e.m. (vertical bars) $P < 0.05$ given by ** against positive control.

Fig. 20. Comparison of F-actin distribution in human neutrophils treated with STMS and various neutrophil locomotion stimulators.

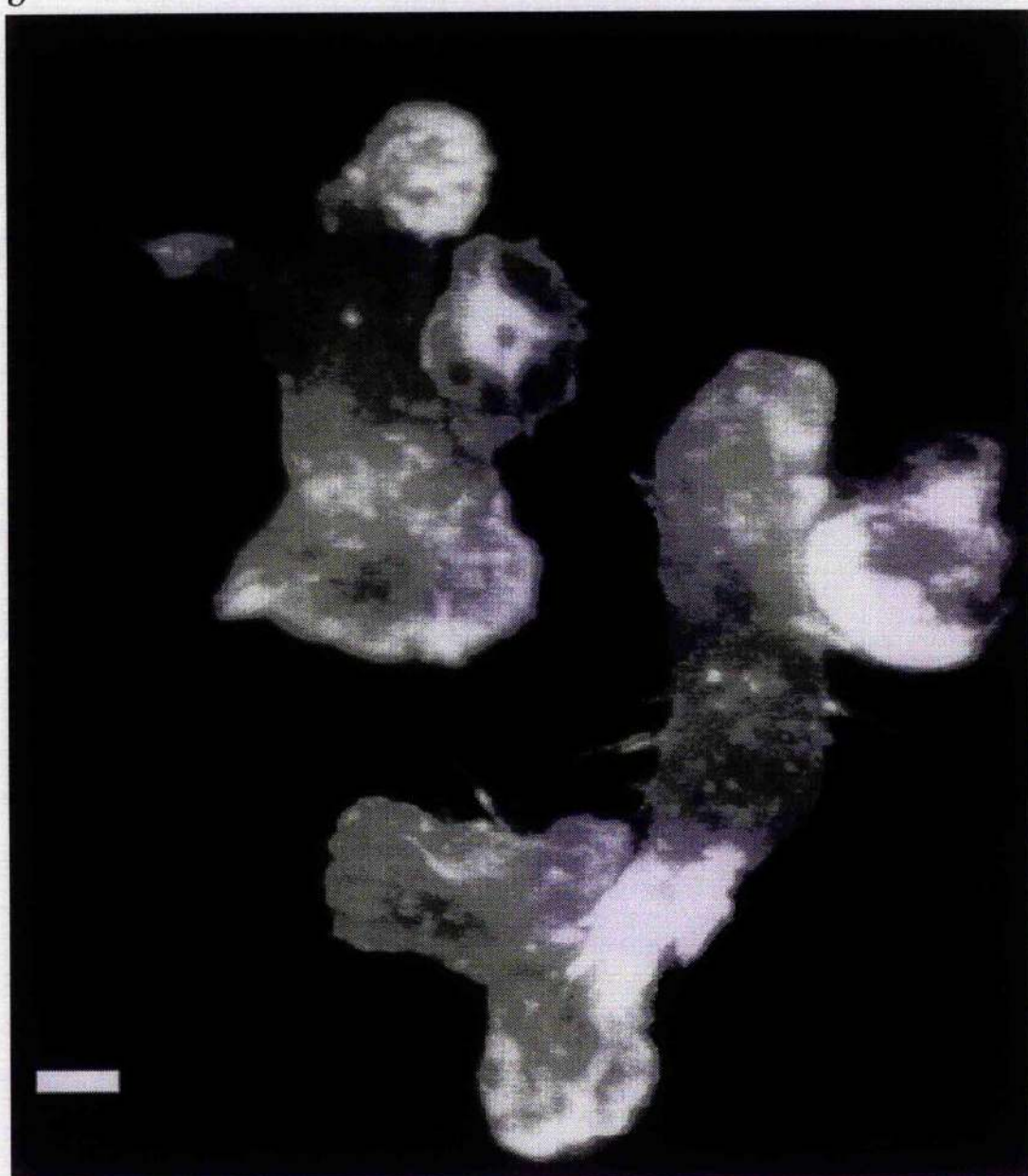
Lighter colour indicates higher concentration of F-actin.

(a) culture medium with and without 10^{-6} M dexamethasone; (b) 10^{-8} M fMLP; (c) CMS; (d) $\text{TNF}\alpha$; (e) STMS. Bar, 2 μm .

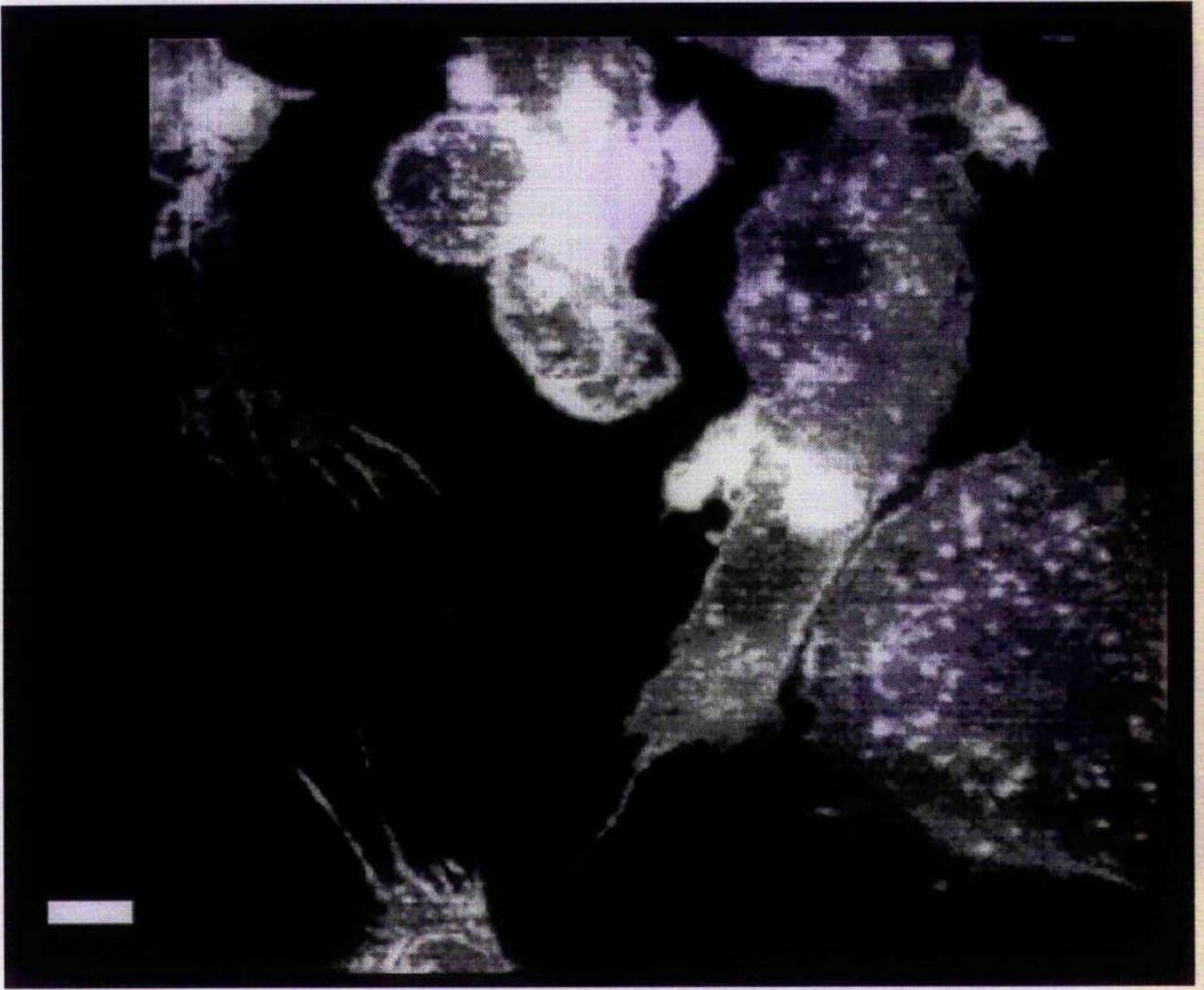
a



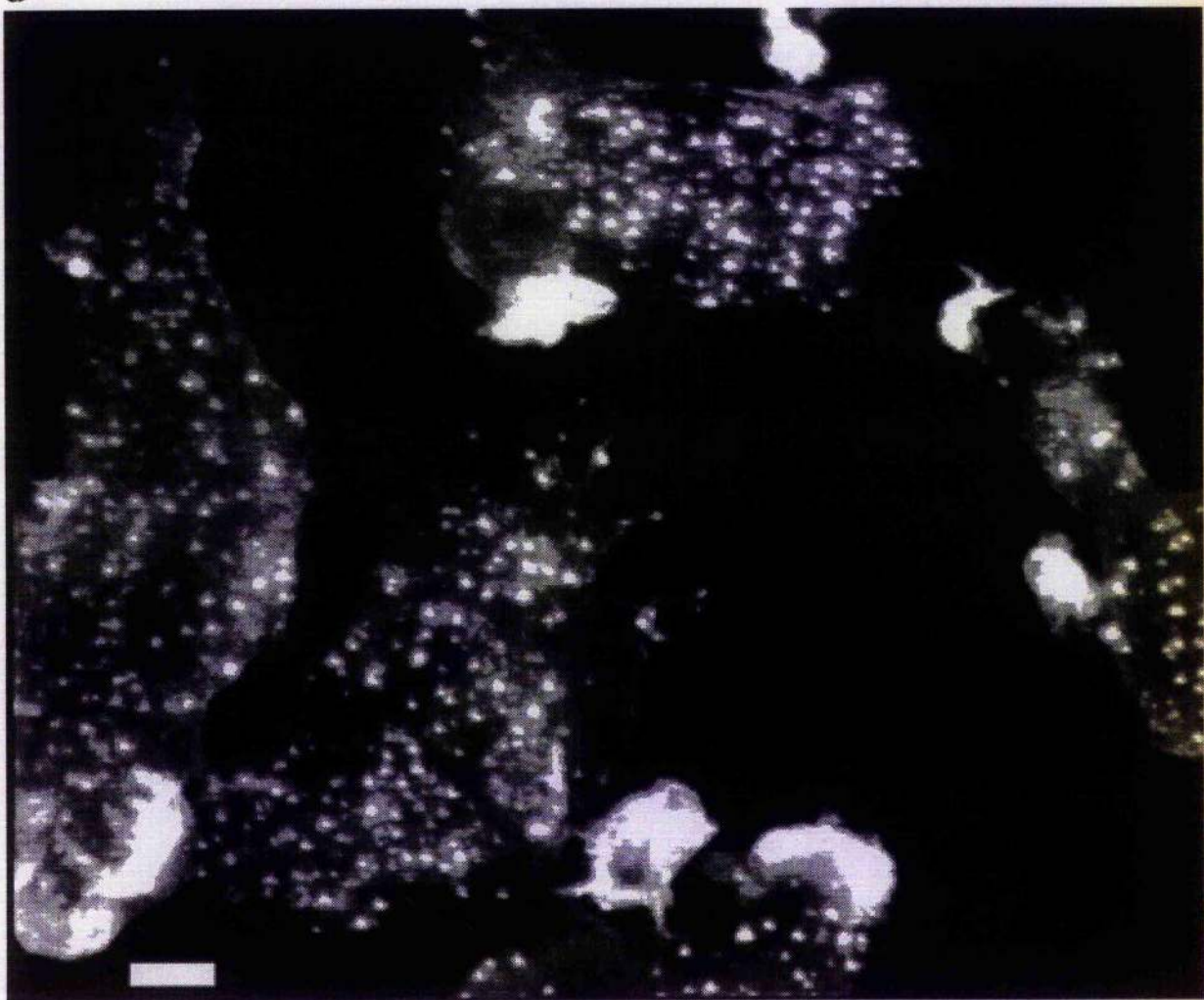
b



c



d



e



in STMS-treated cells was highly unusual and distinct. Staining was only present in the extremes of the bipolar cells and of these two ends, which appear to be points of adhesive contact, one was invariably more intensely stained than the other (Fig 20 e).

3.8.) Purification of the active factor in STMS

3.8.1.) Dose response of starting material compared with that of the partially purified factor

The results of Dr. Chettibi in attempting to purify the active factor from STMS produced a number of puzzling observations. Firstly, there appeared to be two active molecules from the results of gel filtration on Sephadex G30, one of which eluted just before the salt from the concentrated culture medium, the other a larger molecule which eluted along with the larger proteins contained in the supernatant, for example, albumin; 66 kDa. Two regions of activity flanking a region in the centre that showed no activity indicated this. Secondly, he had shown from ultrafiltration that the activity was found to be retained and to go through a small molecular weight cut-off filter. This could also be interpreted as small and large molecular weight active factors.

However, there was another explanation of these results, which could also explain the apparent loss of activity by Dr. Chettibi during previous purification attempts. If there was only one active molecule, of low molecular weight, bound to a larger protein in the starting material, then the puzzling gel filtration and ultrafiltration observations of Dr. Chettibi could be explained. However, they could not explain the loss of activity during purification. If the dose response curve of the purified small

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molecule differed from that of the starting material, then the loss of activity could be explained by assays not being performed at the correct concentrations. The biggest clue to this was on a couple of occasions, finding eluates from the reversed-phase column containing a region without activity flanked by two regions of activity. This indicated that the area in the centre might be active at lower concentration than the flanking regions. These observations would also explain anomalies of the Sephadex G30 gel filtration samples, and in this study, from P2 gel filtration results, in which there was thought to be two active molecules, one of relatively high molecular weight, eluting with the protein fractions and another eluting at the small molecular weight region associated with a pink pigment present in the initial culture medium; vitamin B12 with molecular weight 1351.

Dose response assays were performed on the partially purified factor following gel filtration on Bio-gel P2, and also after the next purification step, of reversed-phase. These showed a 'bell-shaped' dose response curve, one of which is shown in figure 21, with an increase in diffusion coefficient at higher concentrations compared with the control, peaking at one concentration showing STMS levels of diffusion coefficient, and falling off at a lower concentration with a lower diffusion coefficient. The dilutions represented here are from the pooled samples of 50 ml from Bio-gel P2, diluted in HH.

3.8.2) Purification of a molecule of molecular weight 1331 Da, present in STMS

Reducing the volume of starting material was a crucial step in the purification of the active factor in STMS, as the culture supernatants,

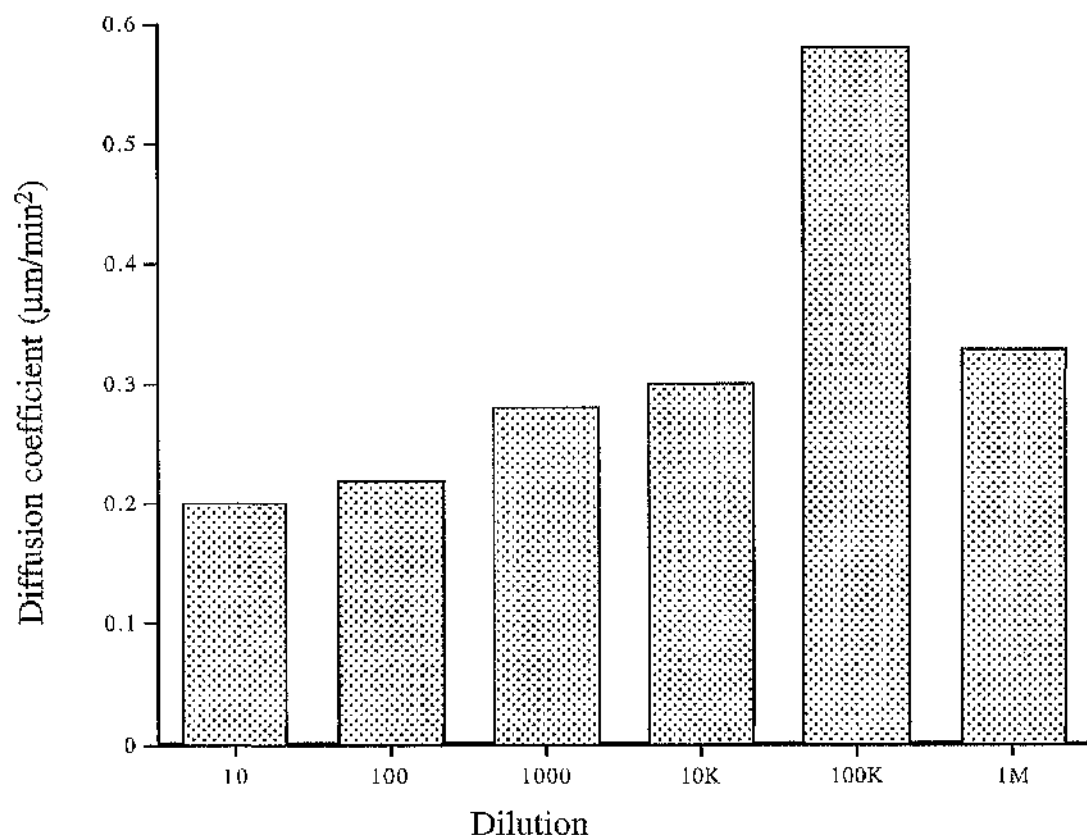


Fig. 21. Dose response of active samples from Bio-gel P2.

Samples from bio-gel P2 were pooled into approximately 50 ml aliquots, and assayed at various concentrations, being diluted in Hank's HEPES. The first dilution, 1/10 takes the concentration of the sample back to 500 ml, that of starting material. As can be seen the peak of activity is 1×10^{-4} that of starting material indicating an increase in the activity of the sample, possibly due to the separation on P2 of the active factor and the putative carrier molecule. Shown is a single assay as an example of the dose-response curve.

K, 1×10^3 ; M, 1×10^6 .

Results

around 300 ml, were of too great a volume to handle using conventional chromatography techniques. Dr. Chettibi had taken a number of approaches, including ultrafiltration and ion exchange chromatography. The results of these approaches were unsatisfactory, as ultrafilters were unable to retain all of the activity, with apparently half being retained and half going through. In order to get the activity to bind to a MonoQ anion exchange column, the sample had to be diluted around ten-fold. This meant that 500ml starting material was diluted in water to 5 Litres before being applied to the column. The limit of speed of the pump meant that it took around 48 hours to complete the 3 Litres, introducing the possibility of bacteria or fungal contamination and/or degradation of the active molecule.

A different approach was taken initially in this study to facilitate the concentration of starting material to a more convenient volume. Approximately 500ml of STMS was processed at a time. This was concentrated by freeze-drying down to around 50ml. This caused some denaturing of protein, and salt precipitation in the sample, which were removed by centrifugation at 3660 rpm for 20 minutes followed by filtration through a 0.2 μ m Millipore filter. This gave a sample of around 40 ml which was then applied to a 500ml Bio-gel P2 size-exclusion chromatography column, separating the active molecule from large proteins, and the high concentration of salt from the concentrated culture medium. Figure 22 shows a UV trace from this separation procedure. The active fractions co-eluted with a region which also contained VitaminB12 (cyanocobalamin), a pigmented molecule (pink) (Fig. 23) of molecular weight 1351 Da, which is present in the Ham's F10 culture medium (indicated by shading).

Fig. 22. Separation of activity from high molecular weight proteins and salt contained in the culture medium

50 ml sample of freeze-dried STMS (500 ml), was applied to a 500 ml bio-gel P2 size-exclusion chromatography column. A 150 mM sodium chloride elution buffer was used to prevent ionic interaction of sample with the gel matrix.

A flow rate of 1.5 ml/min was used; V_o was approximately 100 ml, and V_t , measured by salt elution, was approximately 350 ml.

UV absorption was measured at 254 nm, and sensitivity was 1.0 Absorption units full scale (AUFS).

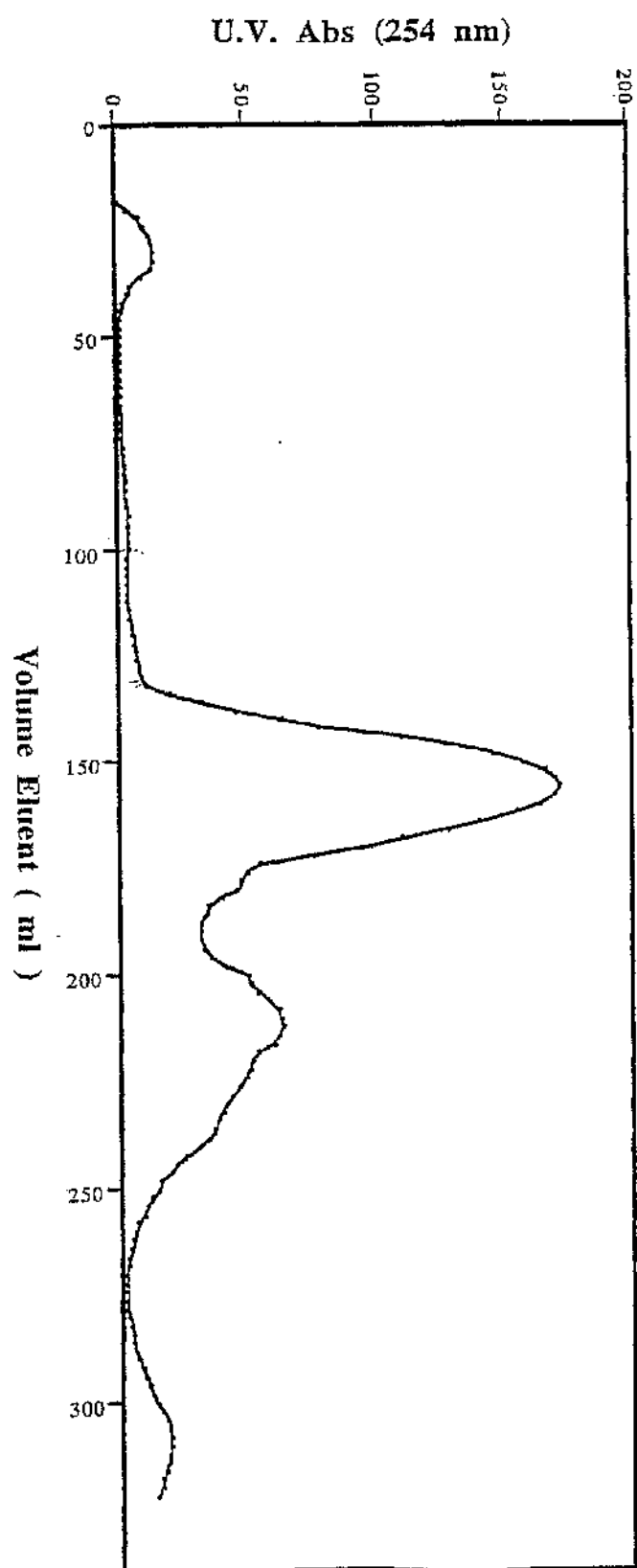
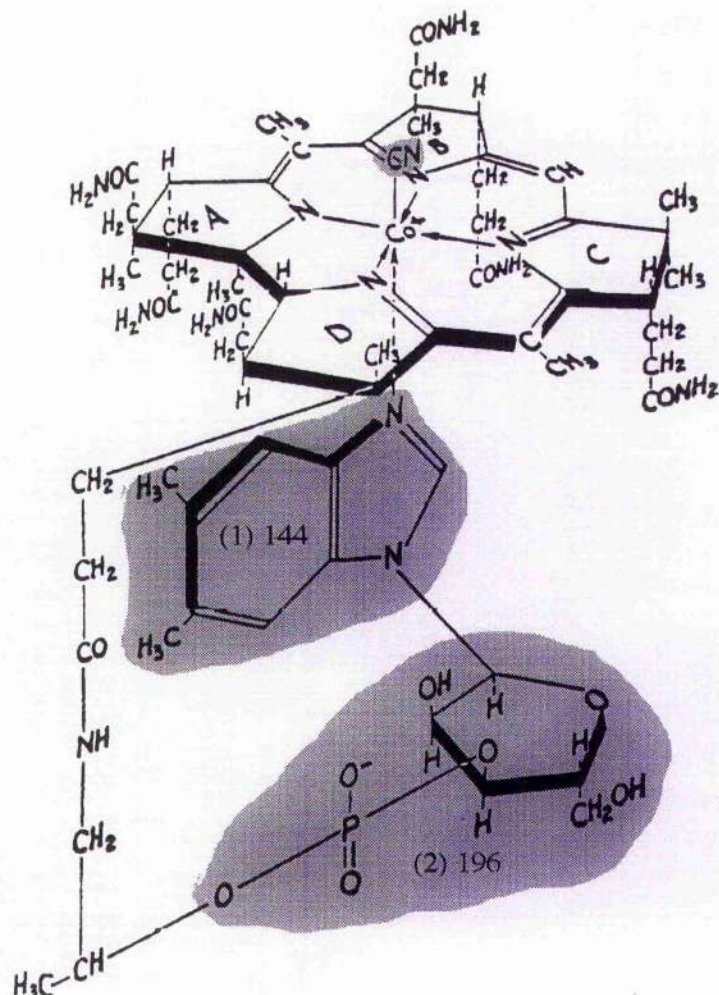


Fig. 23. Diagram showing Vitamin B12 (cyanocobalamin).



From The MERCK index, 10th edition, Merck and Co., Inc., Rahway, N.J., U.S.A., of molecular weight 1351, and found in Ham's F-10 culture medium in which the monocytes are cultured to produce STMS. The shaded areas represent the parts of the molecule removed by high energy Maltetof mass spectroscopy, yielding fragments of molecular weight, 1331 (-CN group), 1188 (-shaded area (1)), and 980 (-shaded areas (1) and (2)).

Results

The active region from the P2 column was spread over a volume of 60-80 ml. The pH was reduced to around 3 using 5M HCl, and the sample was applied to a Pharmacia Sephasil C18 reverse-phase column, using the 50ml injection superloop. Elution with a 0-50% Acetonitrile gradient over 30 minutes separated the activity (shaded area) from the main peak of Vitamin B12 (Fig. 24), but also showed that there were a large number of other molecules present in the sample. The active samples from the reversed-phase column were dried in a vacuum centrifuge in a 1.5 ml eppendorf tube, to around 250 μ l. This was applied to the superdex column at a flow of 500 μ l/min. V_0 of the column was found to be 10ml. Figure 25 shows the UV trace produced from one such sample. The activity was found by tracking assay to be in a region of molecular weight 1000-2000 Da, and when vacuum-dried was found to have a pink pigment present. To further separate the molecules contained in the active region, several types of chromatography were attempted, but with limited success.

Analysis of the active factor following reversed-phase chromatography using Matrix assisted laser desorption time of flight (Malditof) mass spectroscopy by the laboratory of Prof. Rammage confirmed that there was a molecule of molecular weight 1334 in the sample (Fig. 26), one that had previously shown up in samples during Dr. Chettibi's purification. Knowledge of the dose response curve of the partially purified molecule, indicated that this sample would contain more active material, as previously only the periphery of the active molecule had been collected. A sequencing attempt was made on this sample, by Dr. Cronshaw (Wellmet Protein Characterisation Facility, Edinburgh), assuming that there had not been enough material to detect any sequence on the previous occasion. Unfortunately no sequence was detected apart from an initial Alanine residue, and some faint amino acids (results not

Fig 24. Active region from P2 (Fig. 22) applied to a Pharmacia Sephasil C18 reversed-phase chromatography column.

The pH of the sample was reduced to 3, and applied to the column using the Pharmacia superloop, and eluted using a 0-50% buffer B gradient over a 30 minute period at 500 ml/min, UV absorbance 214 nm, and 0.2 AUFS.

Buffer A: 0.14% Trifluoroacetic acid

Buffer B: 0.12% Trifluoroacetic acid, 50% Acetonitrile.

150 μ l samples were collected in 0.5 ml eppendorf tubes, dried using a vacuum centrifuge, and redissolved in 100 μ l of water before being assayed.

Active fractions are shaded.

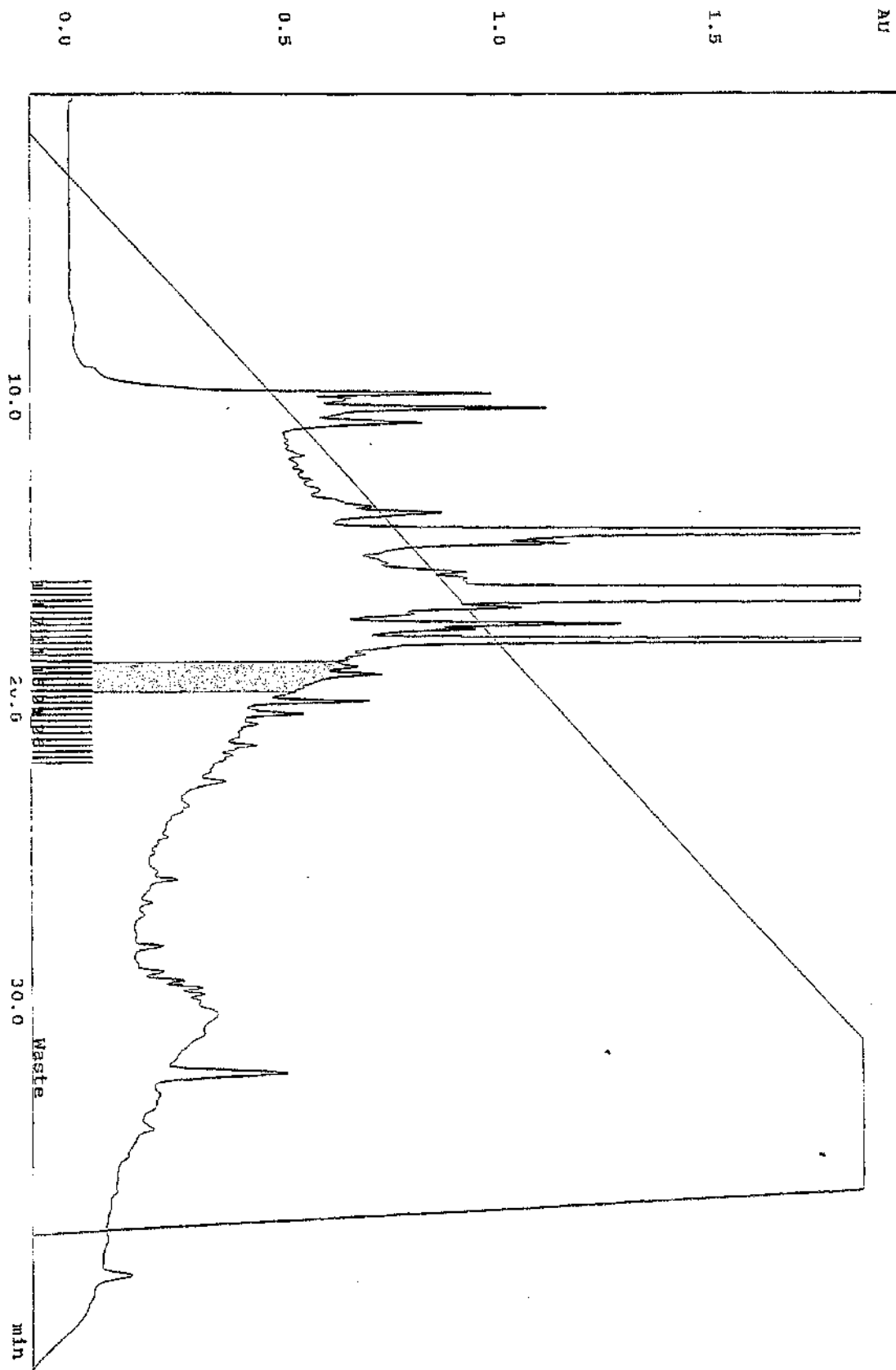


Fig. 25. Active fractions from the reversed-phase purification (Fig. 24) applied to a Pharmacia superdex peptide column.

A 250 μ l injection sample was run at 500 ml/min in 10mM hydrochloric acid, 20% Acetonitrile buffer to reduce ionic interactions of the sample with the gel matrix. Detection was at 0.2 AUFS. V_o was found to be approximately 10 ml, and V_t approximately 20 ml.

Active fractions are shaded.

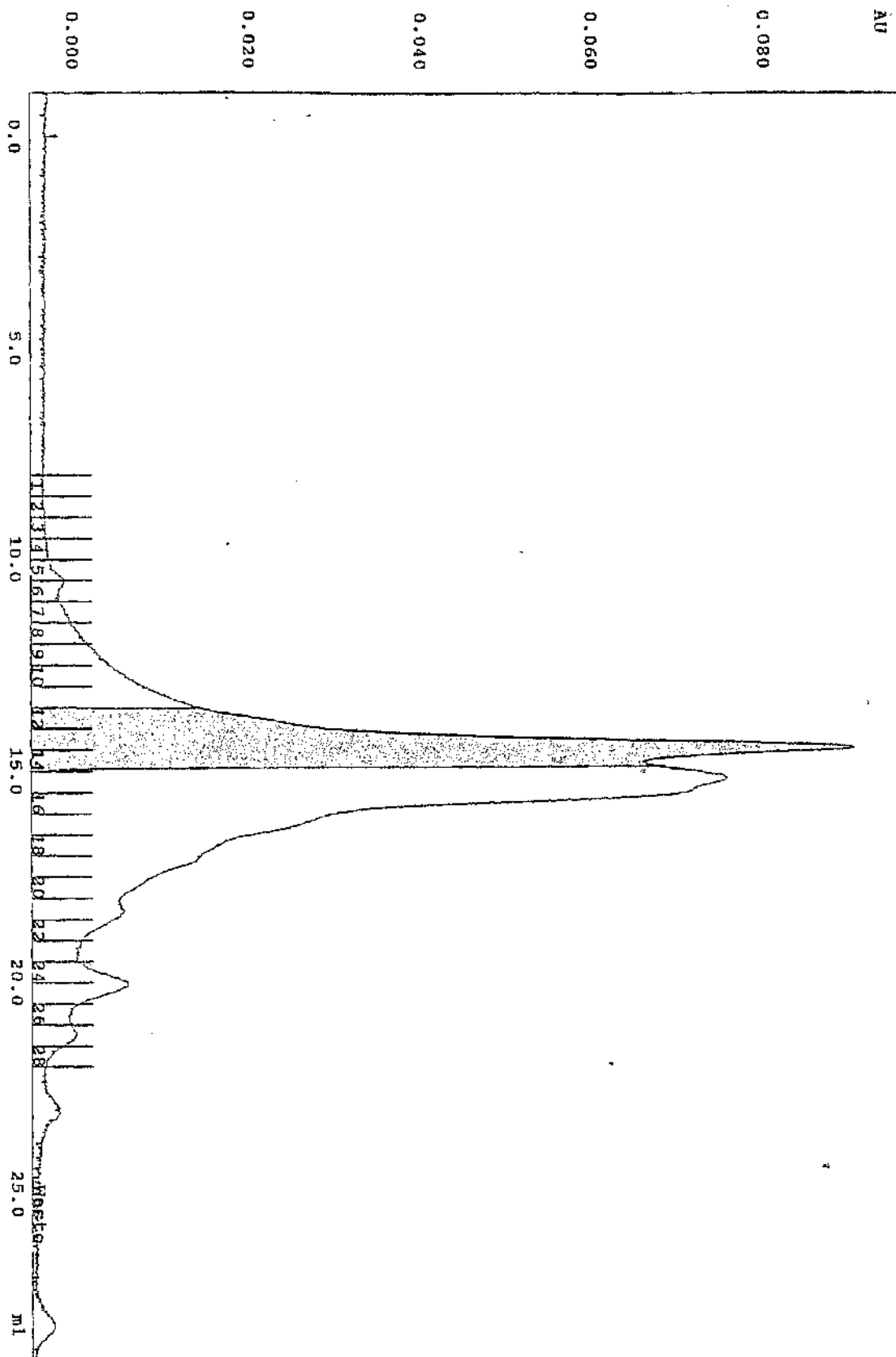


Fig. 26. Matrix assisted laser desorption time of flight (Maldetof) mass spectroscopy of the active fraction from superdex peptide column (Fig 25)

A major peak of approximately 1335 was detected, and is seen alongside the sodium and potassium adducts of the parent molecule (1350.97 and 1366.87, respectively). Another peak of 1296.77 was also detected in the sample.



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Account: R.RANAGIE

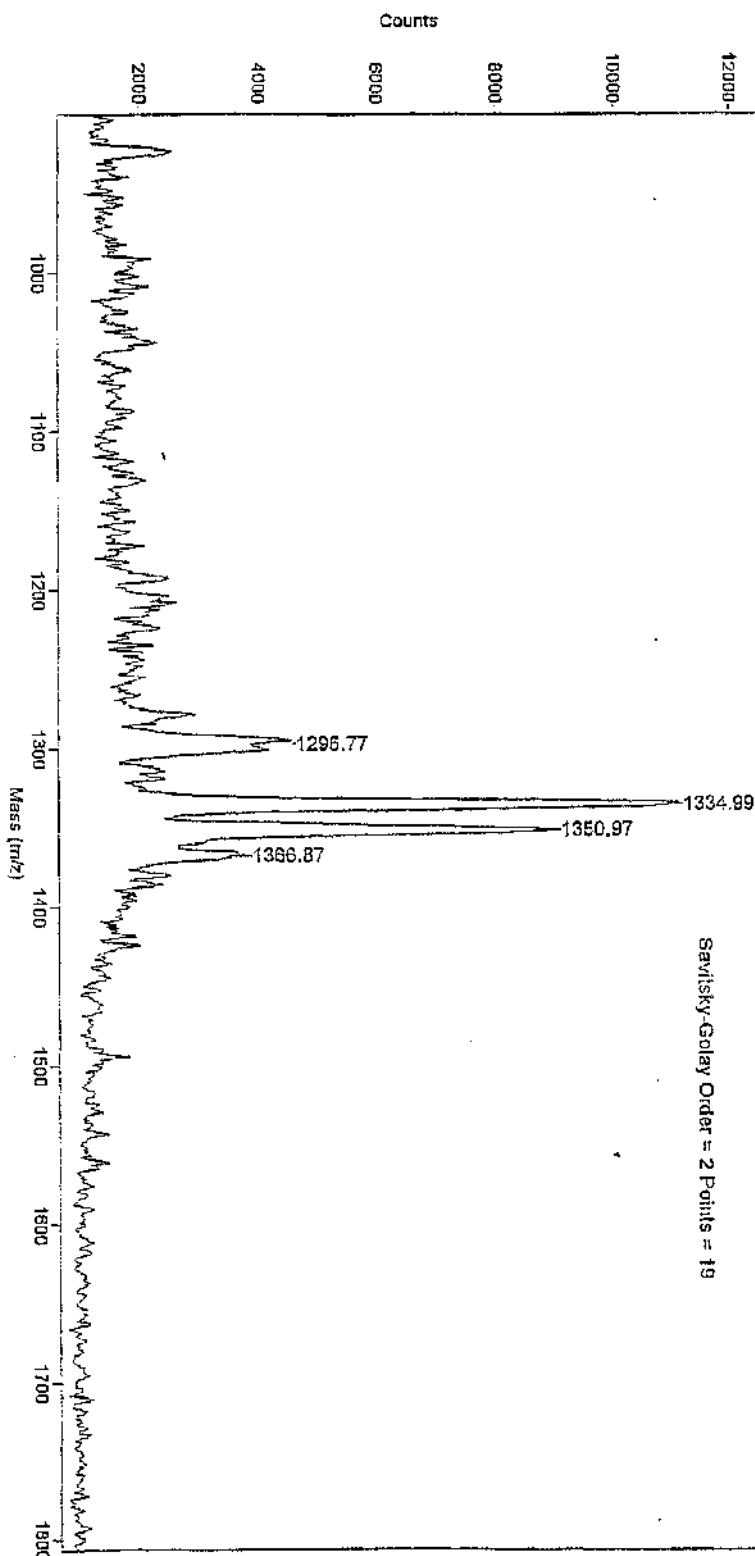
Original Filename: c:\data\sim02a.ms

This File # 4: C:\DATA\SMOOTH.MS

Collected: 7/9/96 4:35 PM

Sample: B6

Savitsky-Golay Order = 2 Points = 19



Comment:

Method: DEFAULT

Accelerating Voltage: 25250

Low Mass Gate: OFF

Negative Ions: OFF

Laser: 700

Scans Averaged: 3

Pressure: 9.52e-08

Results

shown). This indicated that if the molecule was indeed a peptide, it may have a blocked N-terminus, or there was still not enough material to produce a satisfactory sequence.

Dr. Cronshaw suspected that his sequencing apparatus was not sensitive enough to detect the quantities in our sample, so he put us in contact with Dr. G. Kemp (Cell and Molecular Biology, University of St. Andrews), who had sequencing capabilities with an order of magnitude greater detection level. Dr. Kemp attempted sequencing on a number of active samples and surrounding peaks, and found a peptide that corresponded to a fragment of actin, residues 31-41 (WPSIVGRPRHQ). It was found that this was in fact a contamination product as it had mass of 1286, and eluted from the reversed-phase column slightly after the activity. Unfortunately, in the most active fractions there was very little information from conventional sequencing attempts, indicating that if the active molecule was indeed a peptide, then it most probably had a modification of the N-terminal amino acid that prevented conventional sequencing.

Amelia Jackson, from Kratos, a company who manufacture 'Maldi V' mass spectroscopy equipment kindly agreed to analyse our sample on their Maldi IV apparatus, which showed the same peak previously seen, of 1331 MW (Fig. 27). Increasing the laser power during mass spectroscopy allowed them to fragment the 'peptide', producing peaks that correlate with the amino acids that make up the molecule. This yielded some information about the nature of the active molecule. Figure 28 indicates the fragmentation pattern of the active molecule. Analysis of these fragments showed residue fragments of 143, 206.6, 72, 56, and 56. These could be interpreted as Phenylalanine (147.1), followed by a fragment with two amino acids together 206.6, which is a common occurrence in this type of fragmentation (Amelia Jackson, personal communication), followed by alanine (71.0), and two glycine residues

Fig. 27. Matrix assisted laser desorption time of flight (Maldetof) mass spectroscopy by Kratos using Maldi V, of an active sample. A main peak of 1331.3 was detected, with a number of small molecular weight peaks.

Glasgow
Sample 122.JYRP33/Cyano
Data: Glasgow0005.3 13 Nov 96 7:10 Cal: SubPCyano 13 Nov 96 7:00
Kratos Kompact MALDI 4 V5.2.0: + Linear High Power: 91
%Int. 100% = 15 mV (sum = 734 mV) Profiles 1-47 Smooth Av 61 - Baseline 50

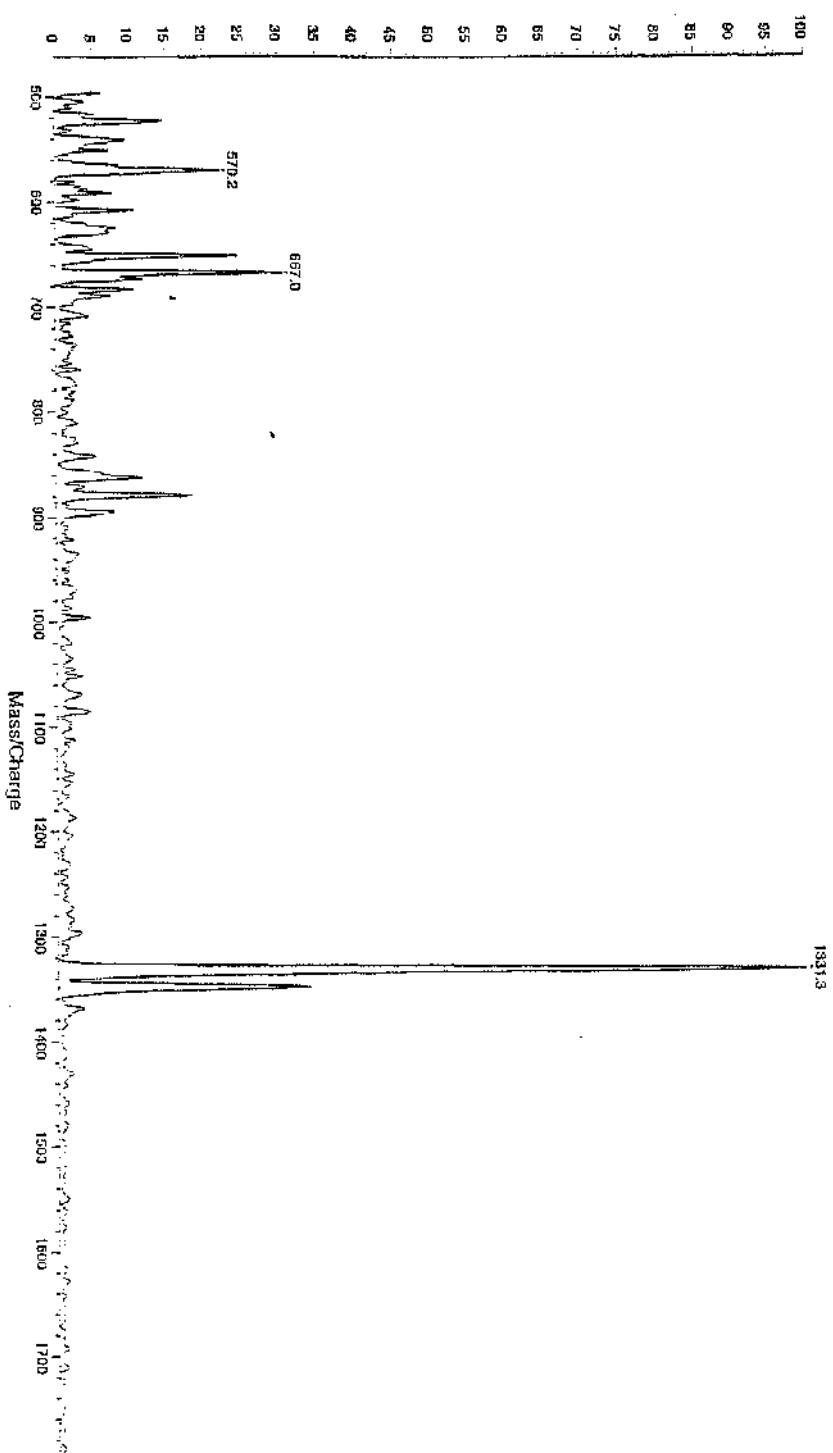


Fig. 28. Fragmentation of 1331 MW peak by mass spectroscopy

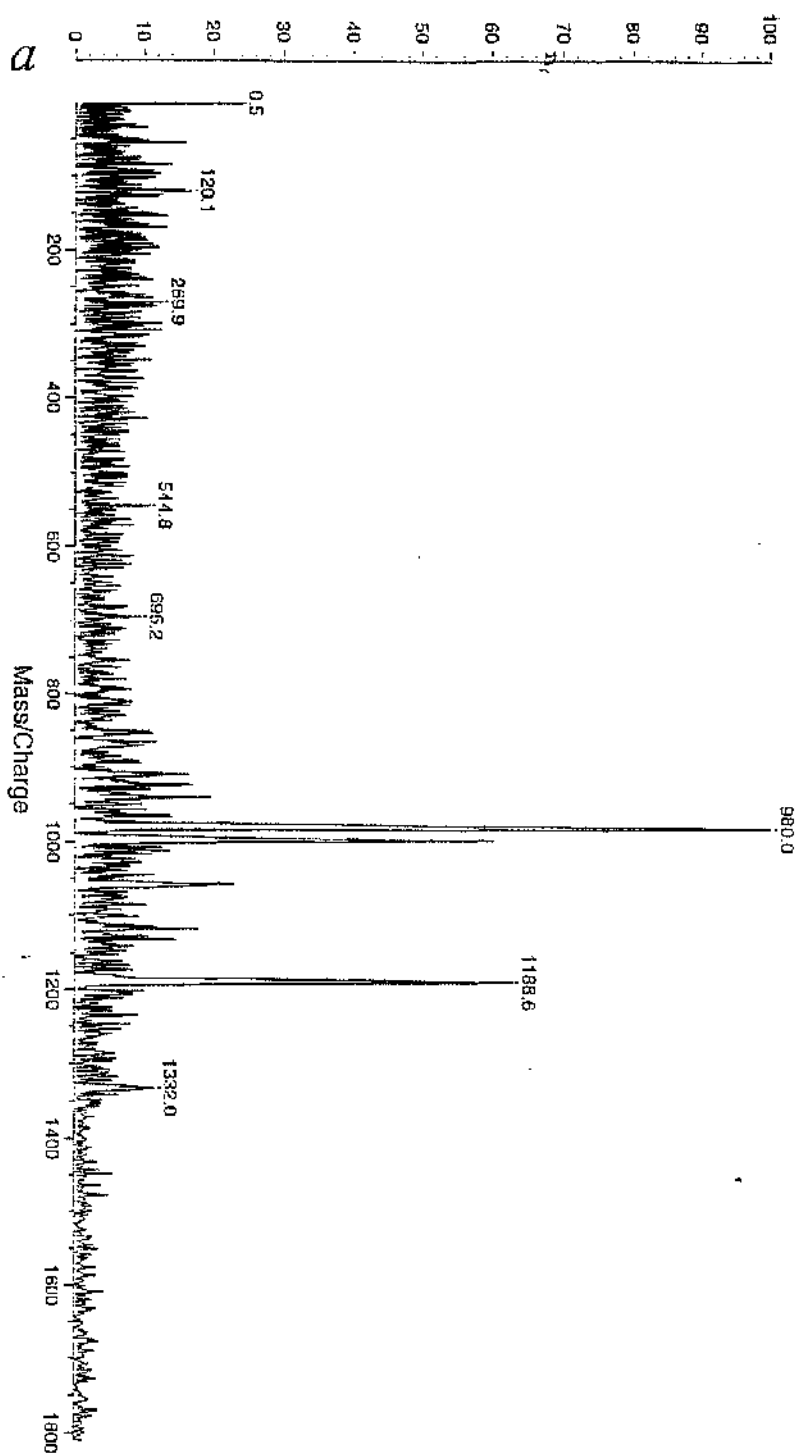
A min peak of 1331.3 was detected, with a number of small molecular weight peaks. Increasing the laser power allowed fragmentation of the parent molecule into constituent parts. Subtraction of fragment sizes allows calculation of the amino acids present in the peptide molecule.

(a). three peaks are present in this fragmentation, 1332.0, which represents the whole molecule, 1188.6, and 980.0

(b). Gating of the mass spectroscope allows only fragments of the 1188 initial fragment to show. This produced several further fragments, 979.2, 907.7, 851.0, 795.0, and 740.1. The lower molecular weight fragments are picked out arbitrarily from the baseline by the processing software.

(c). Gating of the 980 fragment produced fragments of sizes, 991.7 representing the 980.0 fragment, 921.5, 864.7

Glasgow University
Sample STMS Glasgow Cyano
Data: STMS0006.3.1 Jul 96 11:59 Cal: SubP 1 Jul 96 11:46 (Frag: 1332.04)
Kratos Kompact MALDI 4 V5.1.2: + Reflection High Power: 83, Gate: 1310.00-1351.00
%Int. 100% = 25 mV/sum = 1775 mV] Profiles 0-70 Smooth Av 10 - Baseline 80



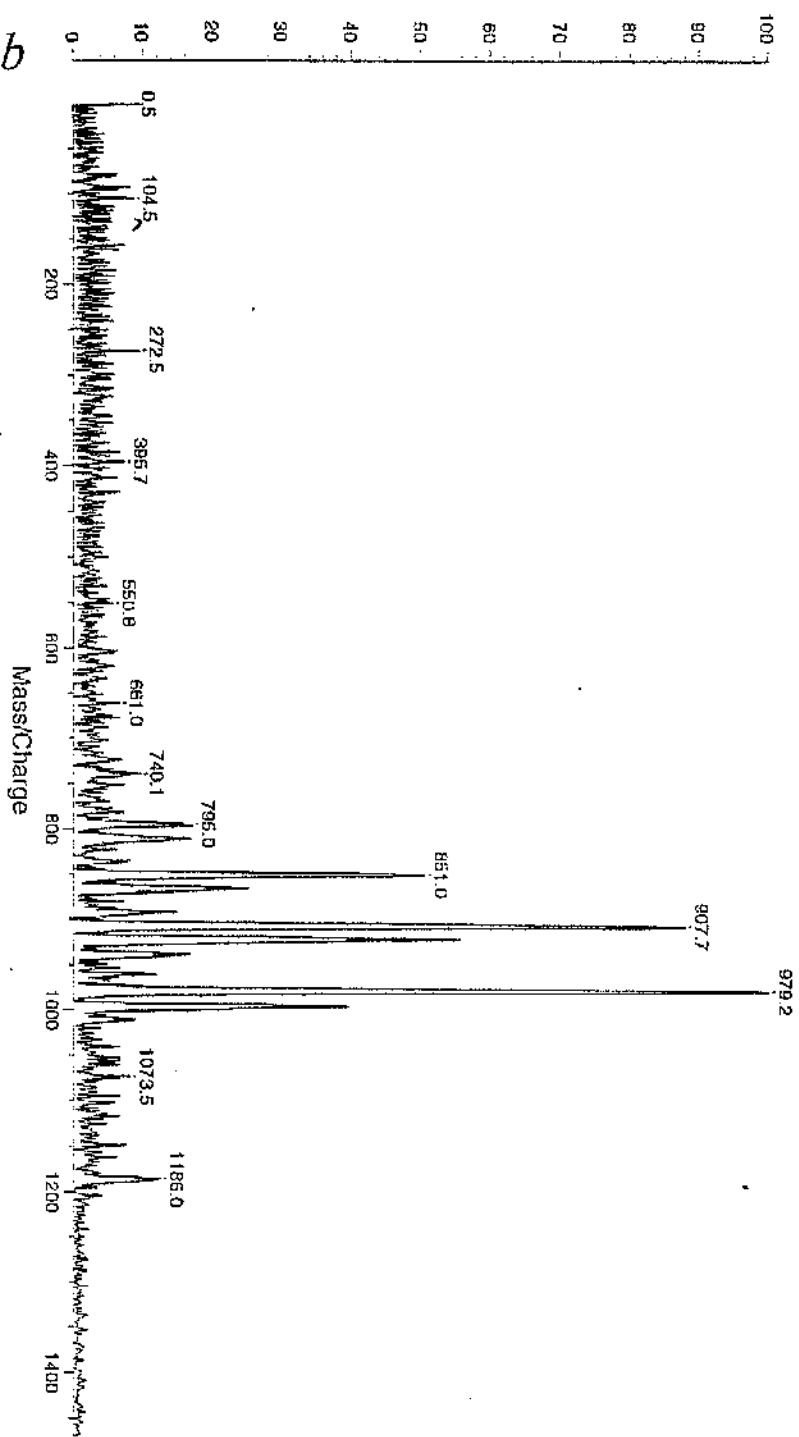
Glasgow University

Sample STMS Glasgow Cyano

Data: STMS004.3 1 Jul 96 11:55 Cal: SubP 1 Jul 96 11:46 (Frag of 1186.11)

Kratos Kompact MALDI 4 V5.1.2: + Reflection High Power: 54, Gate: 1171.00-1216.00

%Int. 100% =23 mV[sum= 4975 mV] Profiles 0-208 Smooth Av 10 -Baseline 80



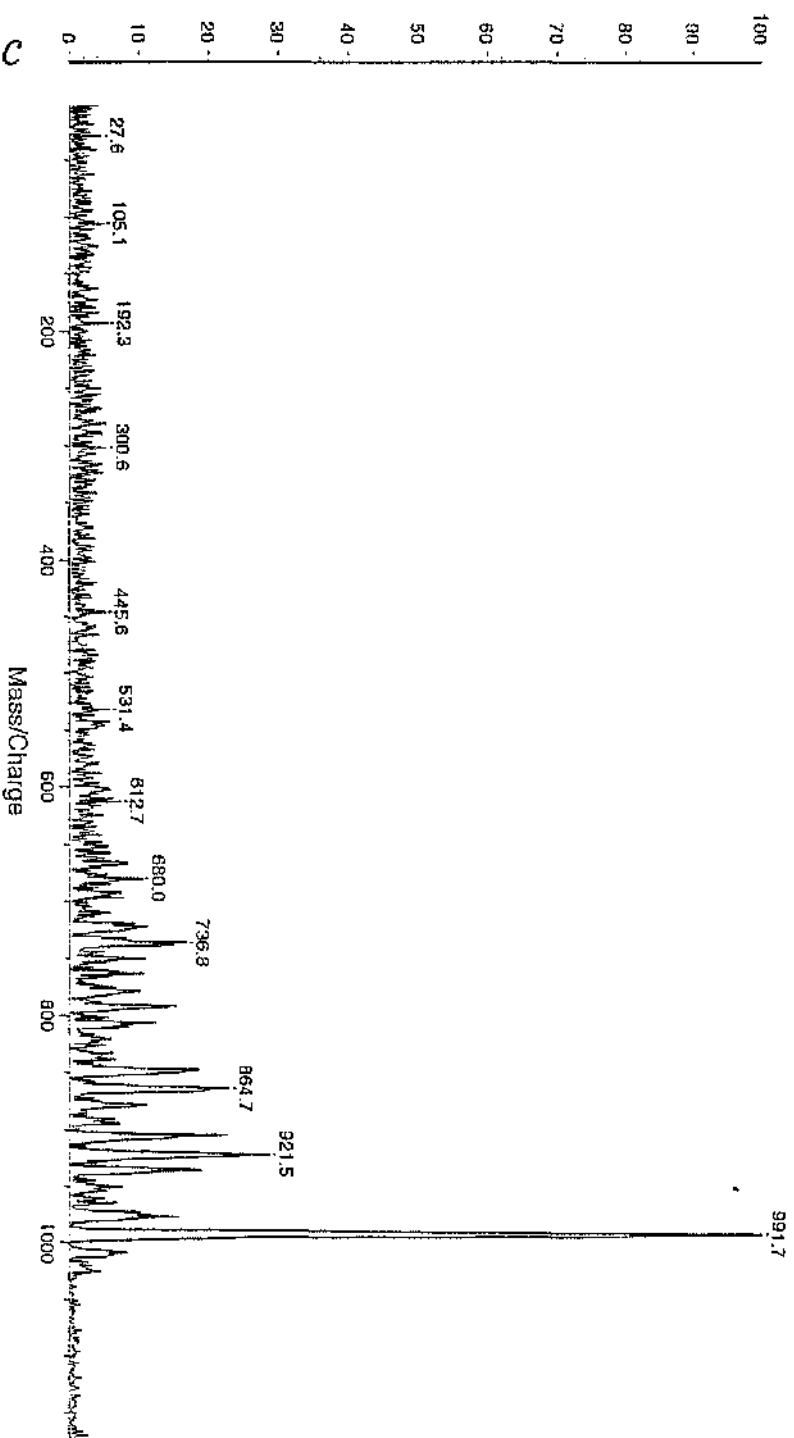
Glasgow University

Sample STMS Glasgow Cyano

Data: STMS0002.3 1 Jul 96 11:52 Cal: SubP 1 Jul 96 11:46 (Fragments of 991.81)

Kratos Kompact MALDI 4 V5.1.2: + Reflection High Power: 66, Gate: 969.00-1022.00

%Int. 100% = 57 mV (sum = 4618 mV) Profiles 0-80 Smooth Av 10 - Baseline 80



Results

(57.0). Unfortunately, there was only partial fragmentation of this molecule, so we were unable to determine the full amino acid sequence using this technique.

3.8.3.) Identification of molecule with molecular weight 1331 Da

At this stage we contacted Dr. Darryl Pappin (Imperial Cancer research fund (ICRF), London) who performed further mass spectroscopy analysis on this molecule. In a short time Dr. Pappin was able to identify this molecule as a derivative of vitamin B12, without a CN group (acyanocobalamin). The fragments in fact correlated with possible fragments from VitB12 (Fig. 23).

To rule out this molecule as the activity Vitamin B12 was assayed and found to have no residual activity (results not shown).

3.8.4.) Purification of Thymosin β 4 with an oxidised methionine residue with Dispersive activity

This information indicated that the active molecule might be present in our assay at very low concentrations. A number of other peaks had appeared on mass spectroscopy analysis but had been ignored due to the strong presence of the 1331 Da molecule. This led to assaying the samples during the early stages of purification at very high dilutions, to determine if most of the active fractions were being discarded, through assaying at too high a concentration. The purification protocol was also altered. As we believed that there may be a large molecule involved in binding the active factor, due to the differences in the dose response curves of the starting material and purified factor, it seemed possible to remove all the

Results

unbound small molecules in the starting material by force dialysis; the activity being retained, bound to the large molecule. The sample was force dialysed *in vacuo*, followed by dialysis against water, before being applied to Mono Q. Active fractions from Mono Q (Fig. 29, shaded area) were then applied to the Pharmacia peptide column in a high salt buffer (300 mM Sodium chloride) to prevent the interaction of the small active molecule with the large carrier molecule. However, this did not occur as the activity remained at the excluded molecular size region (Fig. 30). This was applied to the reversed-phase column and a small peak was found to be active, fraction 18, containing approximately 1.5 μg (when peak area is compared to the elution of a 10 μg sample of thymosin $\beta 4$), in figure 31 a and 33 b, eluting in the same region as previous active samples, away from the majority of proteins which eluted late (Fig. 31 a). Mass spectroscopy analysis by Dr. Pappin, showed this molecule to have a mass of 4980.4, and sequencing of tryptic peptides from this molecule using Low-energy mass spectroscopy (Sherman et al., 1995; Hunt et al., 1986; Wilm and Mann, 1996) yielded six sequences. These were:

Ac-SDKPDMAEIEK	Met oxidised to the sulphoxide (+16 Da),
Ac-SDKPDMSEIEKFDK	Partial cleavage, Met oxidised, Ac- Acetyl
TETQEK	
NPLPSK	
ETIEQEK	
QAGES-COOH	Free acid at C-terminal.

Data base searches revealed the sequences to correlate with the sequence of Thymosin $\beta 4$ with oxidised methionine at residue 6.

Fig. 29. Anion exchange separation using MonoQ of force-dialysed STMS.

STMS was force-dialysed against air to remove small molecules (<10000 MW), followed by dialysis against water to remove any salt from the sample. The sample (approximately 5 ml) was made to pH 8, by addition of 1 ml of 100 mM Tris.Cl pH 8, before being filtered through a 0.22 μ m Millipore filter and applied to the Pharmacia Mono Q anion exchange column in 20 mM Tris.Cl pH 8.0.

A 0-500 mM sodium chloride gradient was applied to the column over 15 minutes, and 500 ml samples were collected in 0.5 ml eppendorf tubes. Fractions were then assayed, and active samples were pooled and prepared for further purification steps.

Activity was found to be in fractions 7-9.

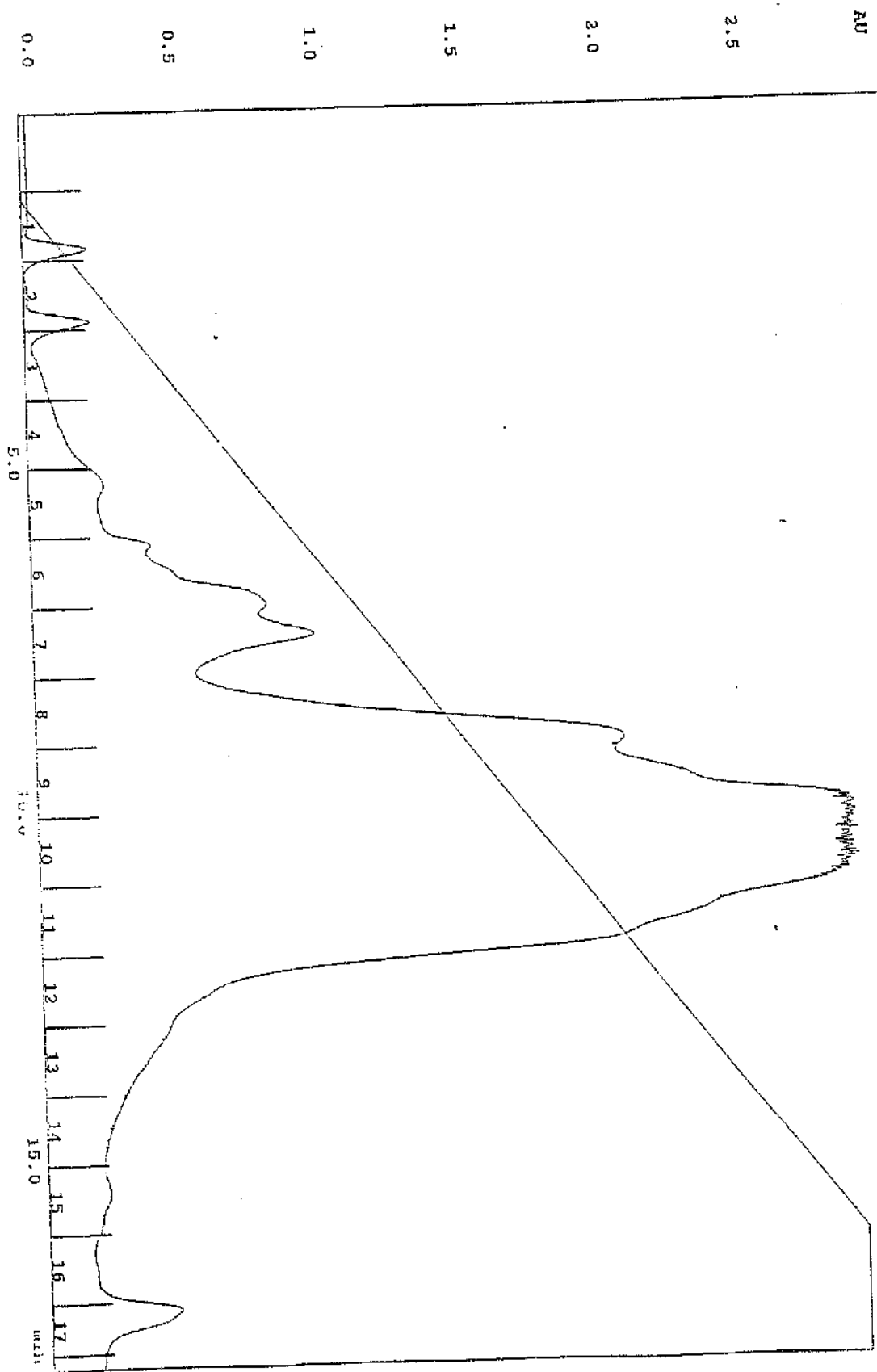


Figure 30. Size exclusion chromatography, using the Pharmacia superdex peptide column

This column was used to attempt separation of the active molecule from the putative large carrier protein.

Pooled active samples from the Mono Q anion exchange column (Fig. 20) were vacuum centrifuged to 250 μ l, and applied to the Pharmacia superdex peptide column in 300 mM sodium chloride, 20 mM Tris. Cl pH 8. The high salt concentration was used to dissociate the active molecule from the putative larger carrier molecule. Activity however, was found to be in the excluded region of the eluted samples (fractions 1-7).

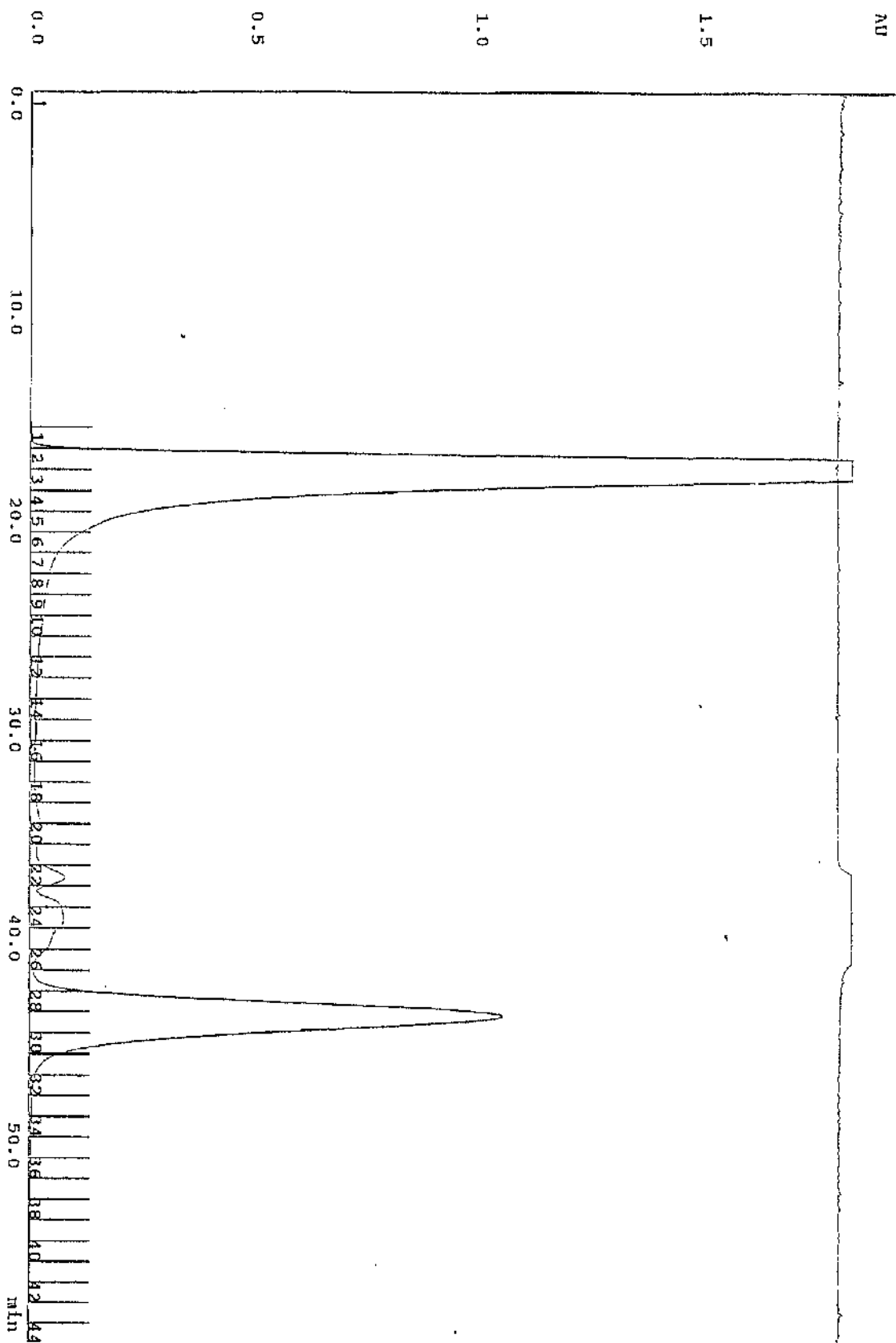
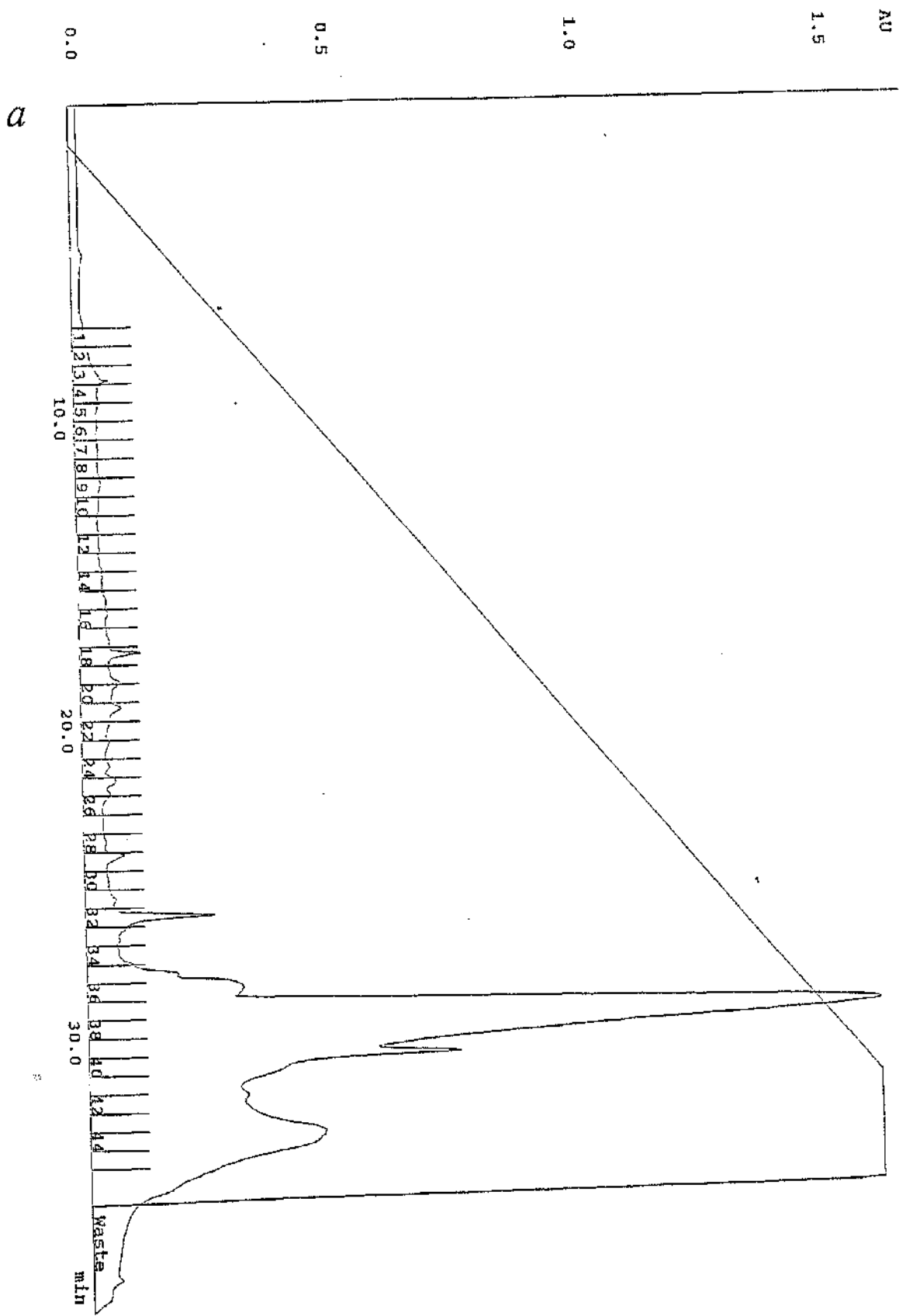


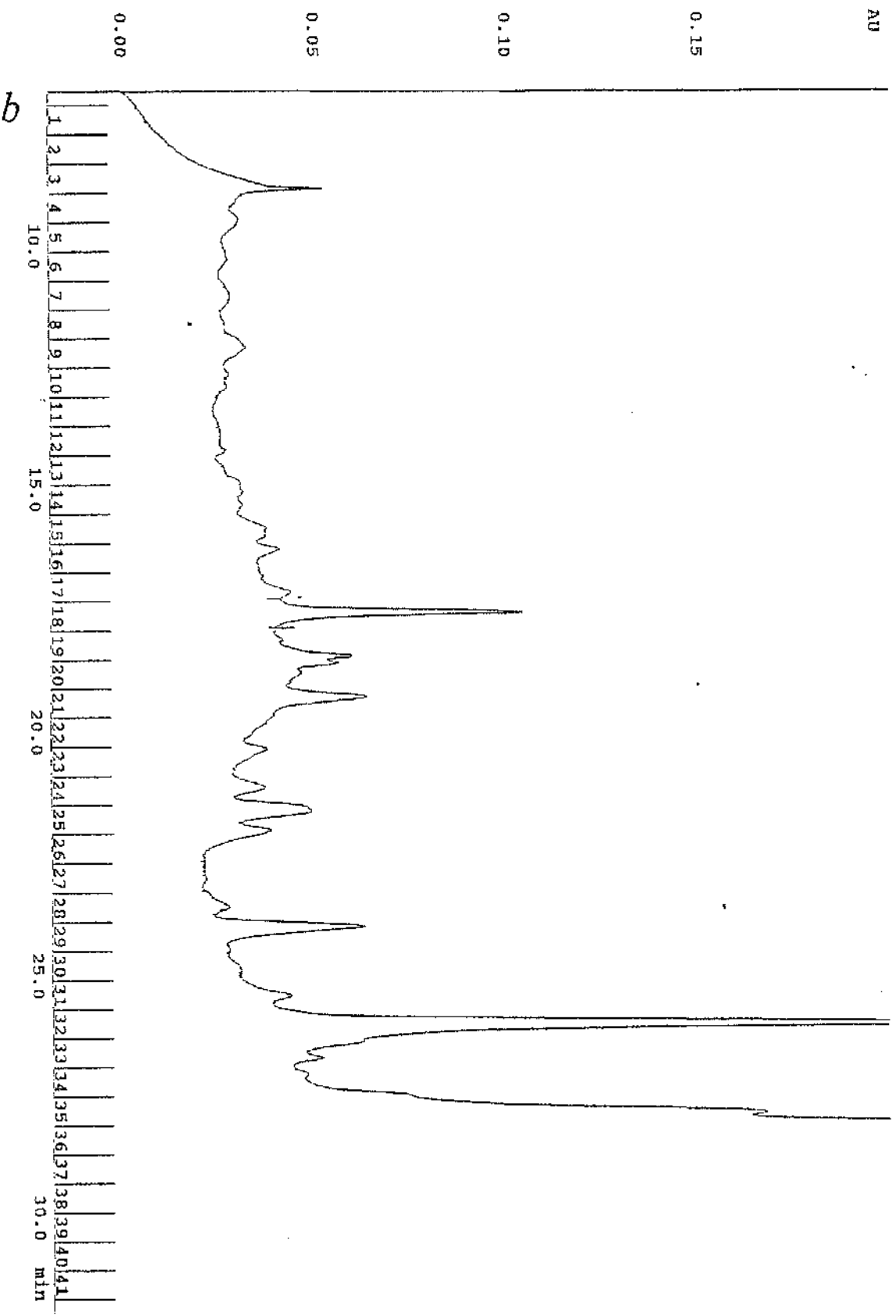
Fig. 31. Active samples from the superdex peptide column applied to the Pharmacia Sephasil C18 reversed-phase chromatography column.

Fractions 1-7 from the peptide column were filtered, using a 0.22 μm Millipore filter, the pH reduced to 3 using 1 M hydrochloric acid, and applied to the Sephasil C18 reversed-phase chromatography column. A 0-50% Acetonitrile, in 0.1% TFA gradient was applied over 30 minutes and 500 μl fractions collected. Samples were vacuum-dried and redissolved in water, before being assayed.

(a). shows the programmed gradient, UV trace, and fractions collected from the reversed-phase column.

(b). A zoom-in to the active fraction 18. This sample was sent for mass spectroscopy analysis.





3.9.) Sequence and secondary structure analysis of Thymosin β 4

Thymosin β 4 (T β 4) was originally purified as a Thymic hormone present in fraction 5 of biologically active thymic extracts (Low et al., 1981). It is an intracellular peptides which regulates actin polymerisation by sequestering G-actin (Cassimeris et al., 1992). It has a single methionine residue, but it is not known whether this is crucial for function. It is also found extracellularly in blood plasma and serum (Naylor et al., 1986; Hannappel and van Kampen, 1987), and is seen in increased levels in the serum of Crohn's disease and ulcerative colitis (Mutchnick et al., 1988). It has been shown to be an important molecule in cell proliferation (Schobitz et al., 1990), differentiation, including immune cells (Shimamura et al., 1990) and cell migration; Malinda et al, 1997, showed that Thymosin β 4 acts as a chemoattractant for Human umbilical vein endothelial cells (HUVEC), and significantly accelerated the rate of migration into the scratch wounded area of a HUVEC monolayer at a concentration of around 1 μ g/ml.

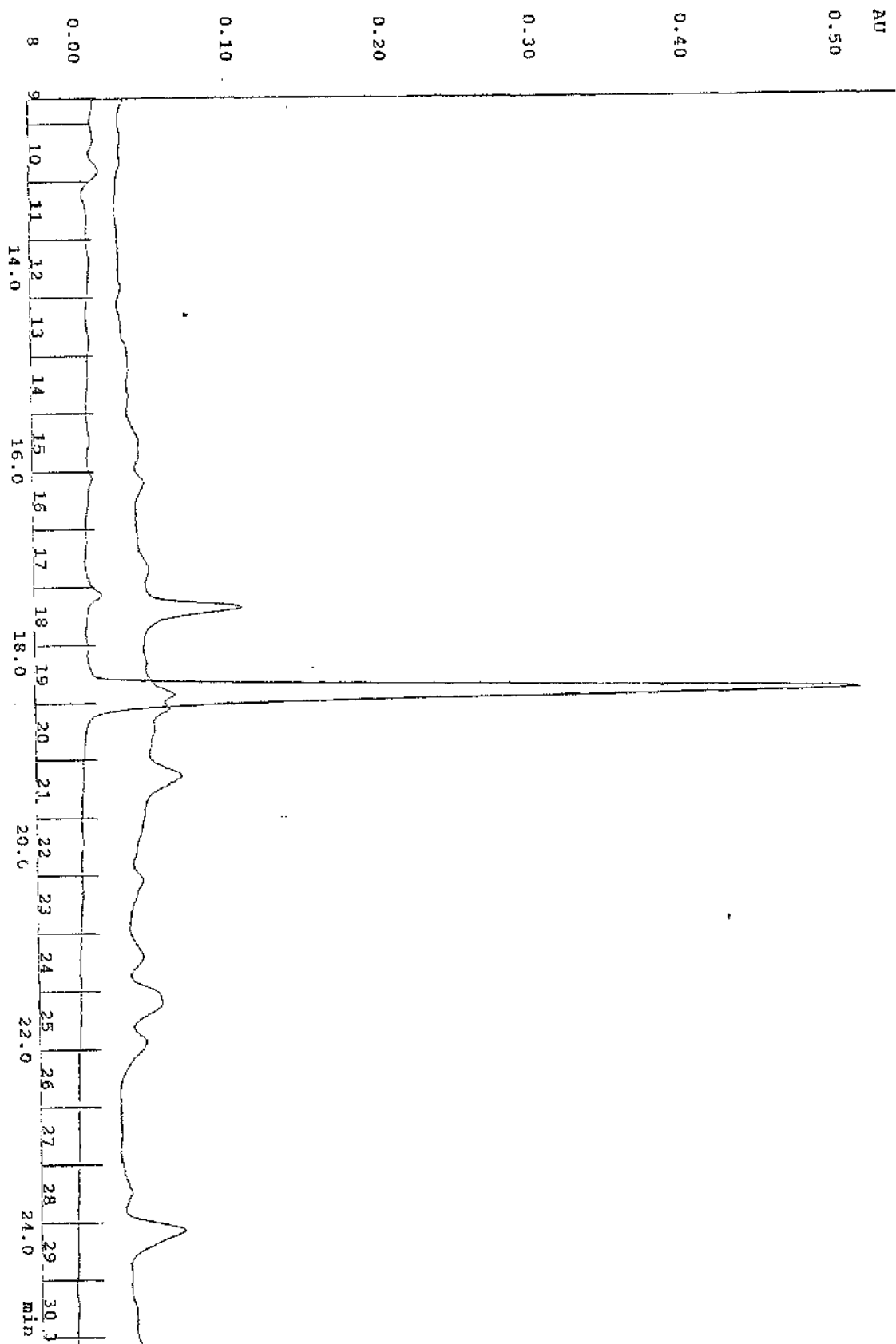
The oxidised peptide may act as a sign of host cell damage and the dispersive response of neutrophils in our assay may be interpreted as an anti-inflammatory strategy.

Interestingly, when the group of Dr. Safer (University of Pennsylvania, USA) provided us with 1 mg of HPLC purified T β 4 from Bovine spleen, we found, by HPLC analysis, that it contained around 10% Thymosin β 4 with the methionine residue oxidised (T β 4ox) (Fig. 32). This led us to speculate that T β 4ox may be active in their assay for scratch wound repair of HUVEC monolayers, and initial results have indicated that T β 4ox is active in this assay at 100x less concentration than native T β 4 (A.G. MacLean, personal communication).

Fig. 32. 10 μ g of Thymosin β 4 eluted from the Sephasil reversed-phase column.

10 μ g from a 1 mg sample of Thymosin β 4 (T β 4) from Dr. Safer, was diluted 1/10 (v/v) in 0.1% TFA and applied to the C18 reversed-phase column, and the same gradient applied as used for previous samples. Comparison of the peak areas indicated that there was approximately 1.5 μ g present in our purified T β 4 with an oxidised methionine (T β 4ox). The 10 μ g sample has been plotted alongside the T β 4ox and as can be seen, there is a shift in the elution from the column caused by the oxidation. The 10 μ g of HPLC purified T β 4 also appears to contain approximately 10% T β 4ox, as seen by the small peak in fraction 18.

This 10 μ g elution was used as a standard for quantitation of T β 4 present in samples eluted from the reversed-phase column.



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Below is the sequence of human, bovine and rat T β 4. As can be seen, there is a single methionine residue at position 6, and a relatively large number of charged E, D and K residues, which may help to explain the ionic interaction of T β 4 with size-exclusion chromatography media. Below the sequence can be seen the secondary structure prediction (BLAST), indicating that the molecule probably contains a number of alpha-helices, and coil structures. The methionine is positioned at the start of the first helix structure, and so its oxidation state may effect the secondary structure of the molecule through disruption of this helix.

```
1 SDKPDMAEIEKFDKSKLKKTTETQEKNP LPSKETIEQEKQAGES human
1 SDKPDMAEIEKFDKSKLKKTTETQEKNP LPSKETIEQEKQAGES bovine
1 SDKPDMVEIEKFDKSKLKKTTETQEKNP LPSKETIEQEKQAGES rat
```

	1	10	20	30	40
T β 4 sequence	SDKPDMAEIEKFDKSKLKKTTETQEKNP LPSKETIEQEKQAGES				
Gibrat method	CCCCCHHHHHHHHHHHHHHHHHHHHHCCCCCHHHHHHHHHHHCCCH				
Levin method	HHHHHHHHHHHHHHHHHHHHCCCCCTHHHCCSCCCCCCHHHHHHHHTHH				
DPM method	CCTCCHHHHHHHHHCCCCCHHHHHHTCTCTCTCHHHHHHHHHHCCC				
SOPMA predict	CCCCCHHHHHHHCCCCCCEEEBCCCCCCCCCCCCCHHHHHHHHHHH				
Consensus	CCCCCHHHHHHHHHCCCCCHHHHHHHCCCCCCCCCHHHHHHHHHCCCH				

Key:

H, G : Helix
E : Sheet
C : coil
S : Bend
B : bridges

3.10.) Comparison of native Thymosin β 4 with the oxidised derivative in neutrophil tracking assays

In order to confirm that the active molecule in our assay was indeed Thymosin β 4 with an oxidised methionine (T β 4ox), and not a contaminant present in very small quantities, or not showing up on mass spectroscopy, it was necessary to purify T β 4 from a different source, unlikely to contain the same contaminants. Initially, using the method of

Results

Hannappel and van Kampen, 1987, Thymosin β 4 (T β 4) was purified from human neutrophils of a single buffy coat, which produced around 20 μ g of pure T β 4 (Fig 33). Analysis by Maldi mass spectroscopy, indicated that both oxidised and unoxidised were present (Dr. Pappin, personal communication), and tracking assays indicated that T β 4ox was indeed active, having the same shape dose response curve, whilst the T β 4 with non-oxidised methionine at residue 6 having a lesser activity at higher concentrations (Fig 34). This left us with strong evidence that T β 4ox was responsible for the dispersive locomotion of STMS. However, because the source of this T β 4 was an immune cell, and purification was on the same reversed-phase column as the STMS sample, it was clear that another source of T β 4 was required, for definitive proof that there was not a contaminating molecule causing the activity.

Three different approaches were taken simultaneously. We received 1 mg of Bovine pancreatic T β 4 from Daniel Safer. At the same time, Dr. Pappin attempted to synthesise *de novo* T β 4. This has proved to be a difficult task, as initially there was a very low yield, and more recently there has been a problem of modifications to the N-terminal serine residue (Darryl Pappin, personal communication).

Another approach was to utilise the fact that there is a shift in T β 4 elution from the reversed-phase column when it is oxidised. 50% of the unoxidised T β 4 from the neutrophil buffy-coat preparation was oxidised and eluted from the reverse-phase column giving a sample of oxidised T β 4 which was very unlikely to contain any contaminants, unless they too underwent the same shift in elution from the column following oxidation (Fig. 35). Assays of this sample have confirmed that T β 4 with an oxidised methionine at residue 6 causes dispersive locomotion in the tracking assay (Fig. 34). Three assays have been plotted together for non-oxidised thymosin β 4 demonstrating the problem of inconsistency of the assay,

Figure 33. Purified Thymosin β 4 from human neutrophils.

The sample purified as described in section 2.12.2.3., was applied to the reversed-phase column and eluted as previously. This yielded 20 μ g, using the area under the peak to compare with the standard 10 μ g T β 4 (Fig. 32). Fractions 1/2, 4,5, and 7 were sent for mass spectroscopy analysis. Fraction 7 was found to contain T β 4, whilst, fractions 4/5 contained T β 4ox.

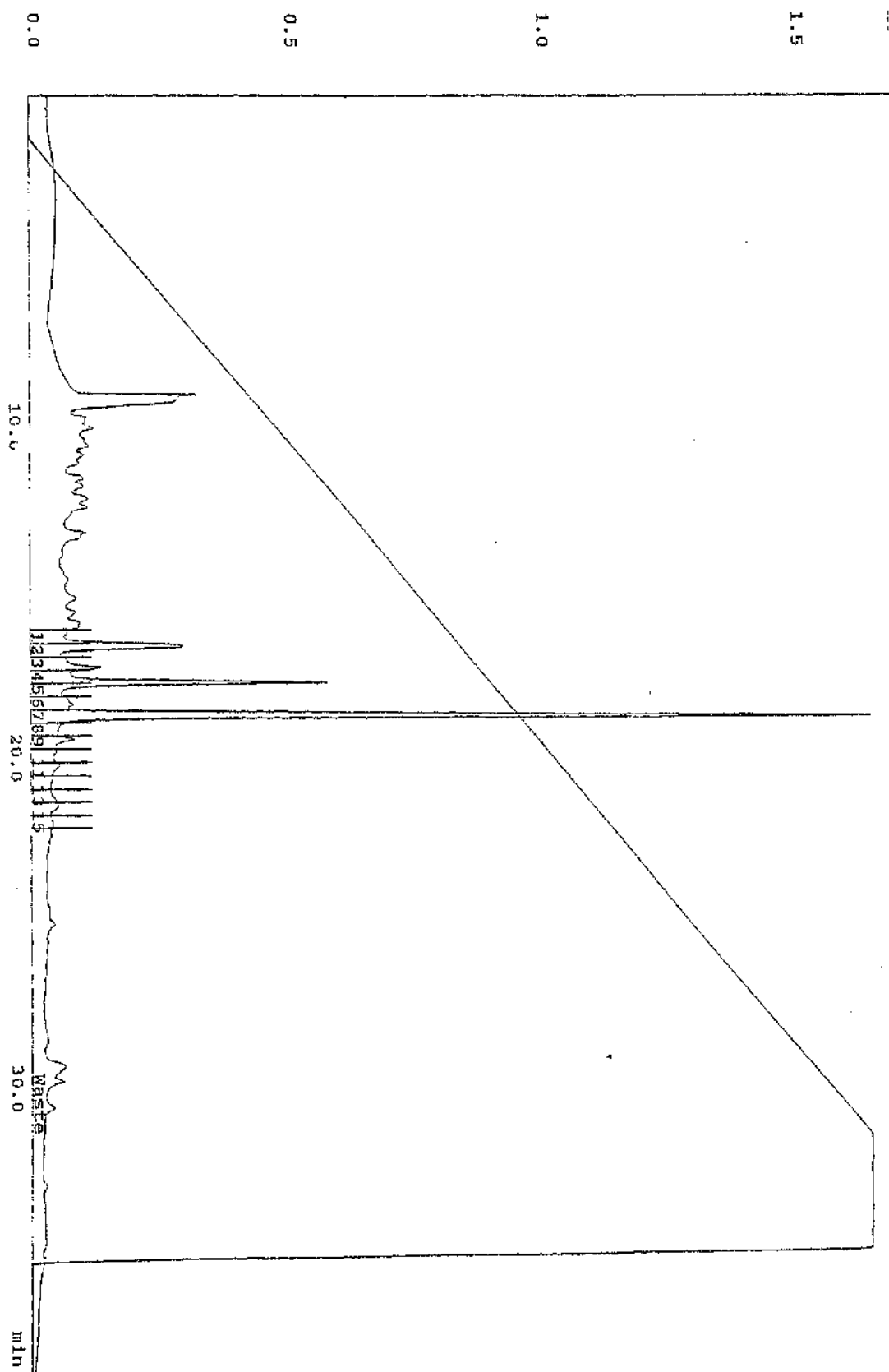







Fig. 34. Comparisons of the diffusion coefficient of neutrophils treated with various concentrations of Thymosin β 4, both oxidised and non-oxidised.

A 60 μ g/ml sample was made for each of the thymosin β 4 samples to be tested. This allowed direct comparison between the oxidised thymosin β 4 (T β 4ox) purified from STMS (60 μ g/ml) and the thymosin β 4 from human neutrophils. Graph (a) shows the results for thymosin with an oxidised methionine residue, purified from STMS , oxidised human neutrophil thymosin β 4 , and hydrogen peroxide oxidised thymosin β 4 from human neutrophils  (b) non-oxidised thymosin from human neutrophils , non-oxidised thymosin β 4 from bovine spleen .

Average diffusion coefficients for the peak of T β 4ox activity were 0.54 ± 0.18 , compared with 0.1 ± 0.04 for non-oxidised thymosin β 4 (T β 4) at the same concentrations. $p < 0.05$; $n = 4$.

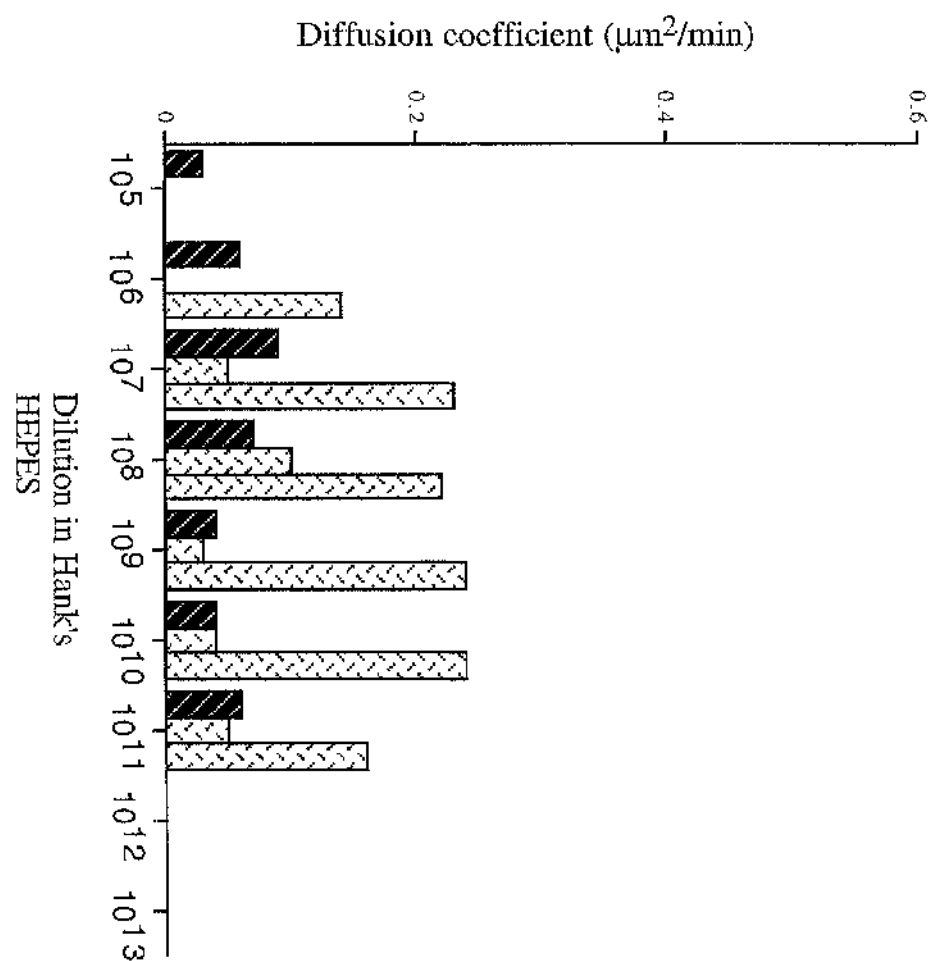
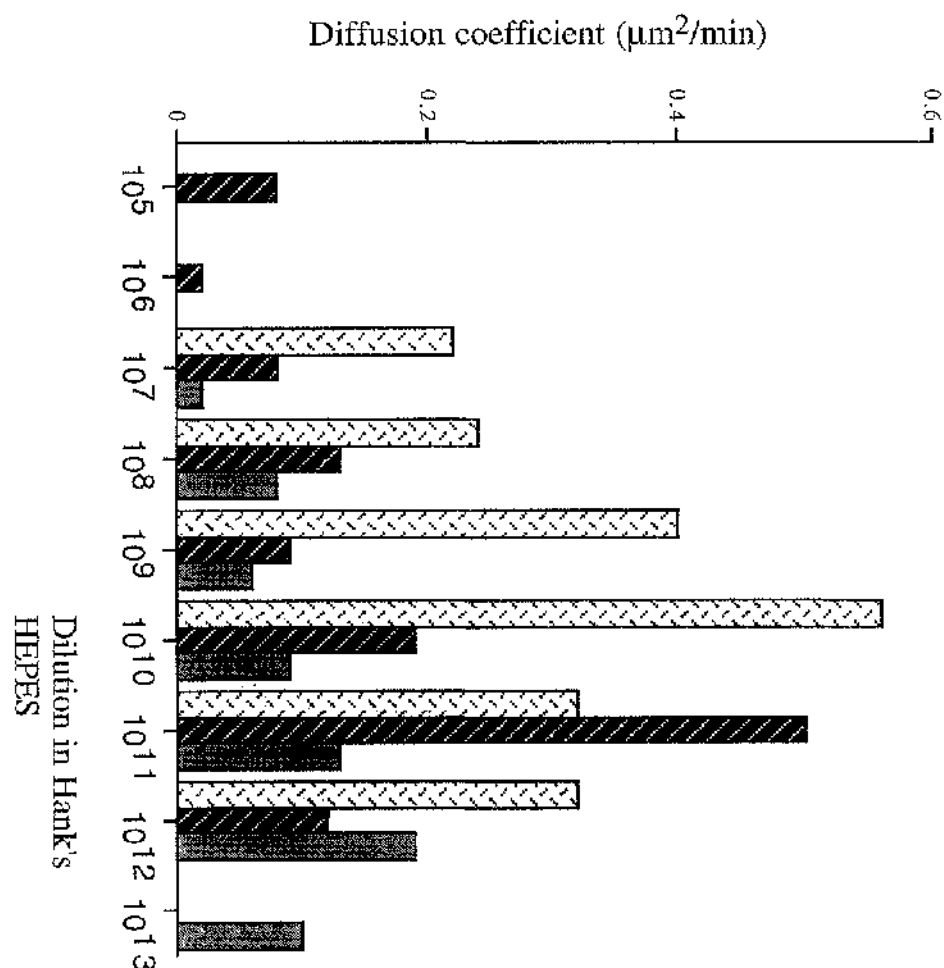
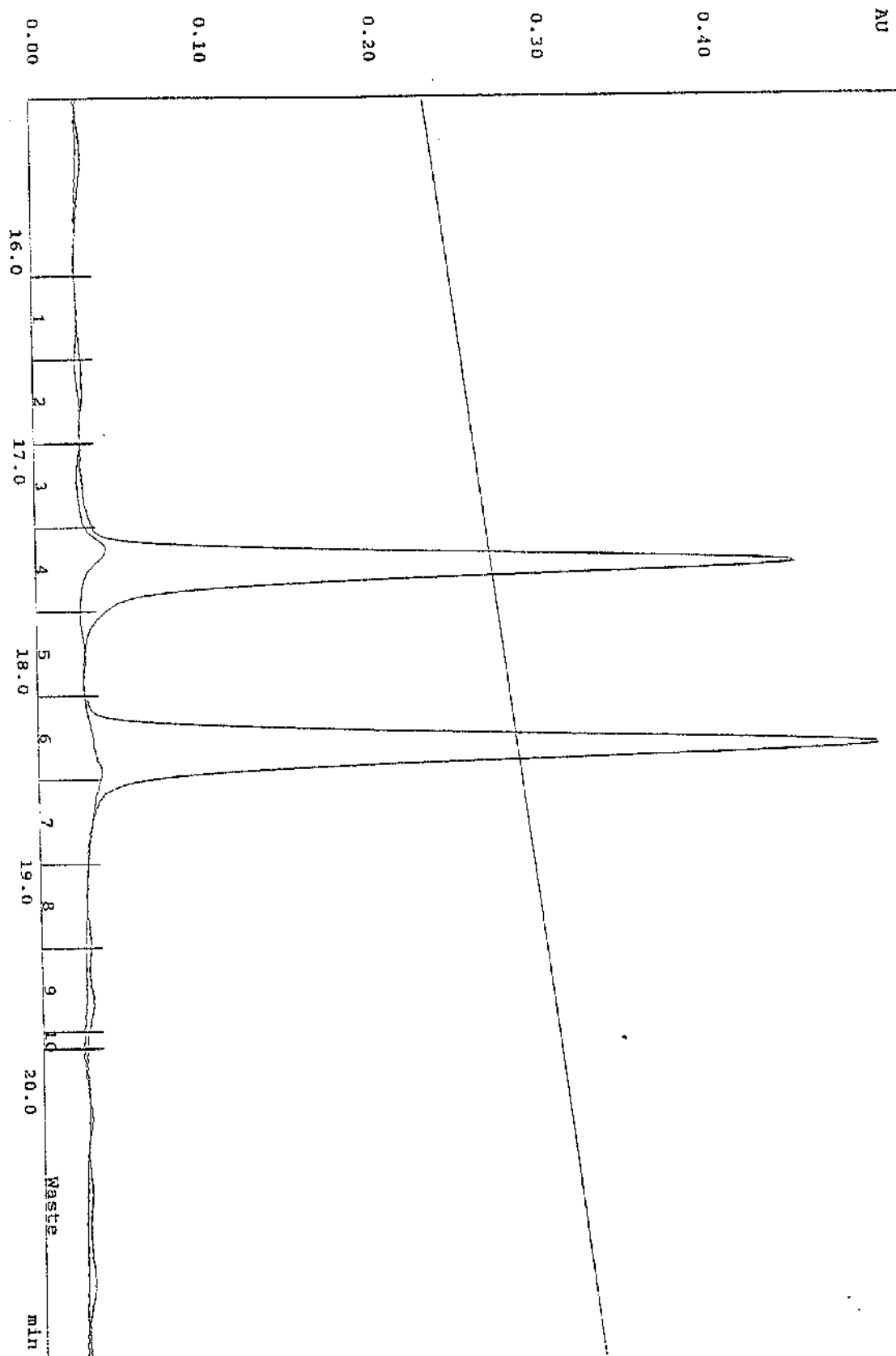


Fig. 35. Oxidation of human neutrophil Thymosin β 4 with hydrogen peroxide and its elution from a reverse-phase chromatography column.

50% of a sample of Thymosin β 4 from human neutrophils was oxidised using the method described in section 2.13. and applied to the Sephasil C18 reversed-phase column. Elution was as previously. The non-oxidised sample was also applied separately. Fraction 6 contains the (50%) unoxidised sample, which elutes as previously, and fraction 4 contains the T β 4ox. As can be seen there is a shift in the elution of T β 4ox, which should separate it from any contaminants from the neutrophil preparation.



with a large variation in baseline activity, as explained earlier in this chapter.

3.11.) Comparison of levels of oxidised Thymosin β 4 in cultured monocyte supernatants with and without steroid.

Parallel purification of cultured monocyte supernatants either in the presence (STMS) or absence (CMS) of dexamethasone, has revealed that T β 4ox is present only in STMS cultures. Two human buffy coat monocytes were cultured in the presence or absence of 10^{-6} M Dexamethasone. This provided approximately 100 ml of both CMS and STMS. These samples were processed through the same purification protocol used to purify the active T β 4ox previously. Figure 36 shows the final reversed-phase analysis of the two samples plotted together. As can be seen, there is a peak associated with the STMS sample that is not present in the CMS sample. Quantitation of these observations, showing the comparative amounts of T β 4 and T β 4ox, using a technique such as, Enzyme-linked immunosorbent assay (ELISA), awaits the production of an antibody specific to T β 4ox.

3.12.) The Lysophosphatidic acid-induced Motility, polarisation and inhibited metabolic burst of Neutrophils.

It has been shown in other laboratories that whilst lysophosphatidic acid has a receptor present on different cell types, none is apparently present on human neutrophils. During investigations into the effects of different activators of neutrophil locomotion, LPA was assayed at biologically

Fig. 36. Parallel purification of Thymosin β 4 from STMS and CMS.

100 ml of STMS and CMS were processed to purify Thymosin β 4, as previously. They were force-dialysed, applied to monoQ, superdex peptide and sephasil C18. The top UV trace shows the STMS sample, with the peak in tubes 3-5 corresponding to T β 4ox. The middle line is that of CMS with little difference between that and the baseline (no sample applied), at the bottom.

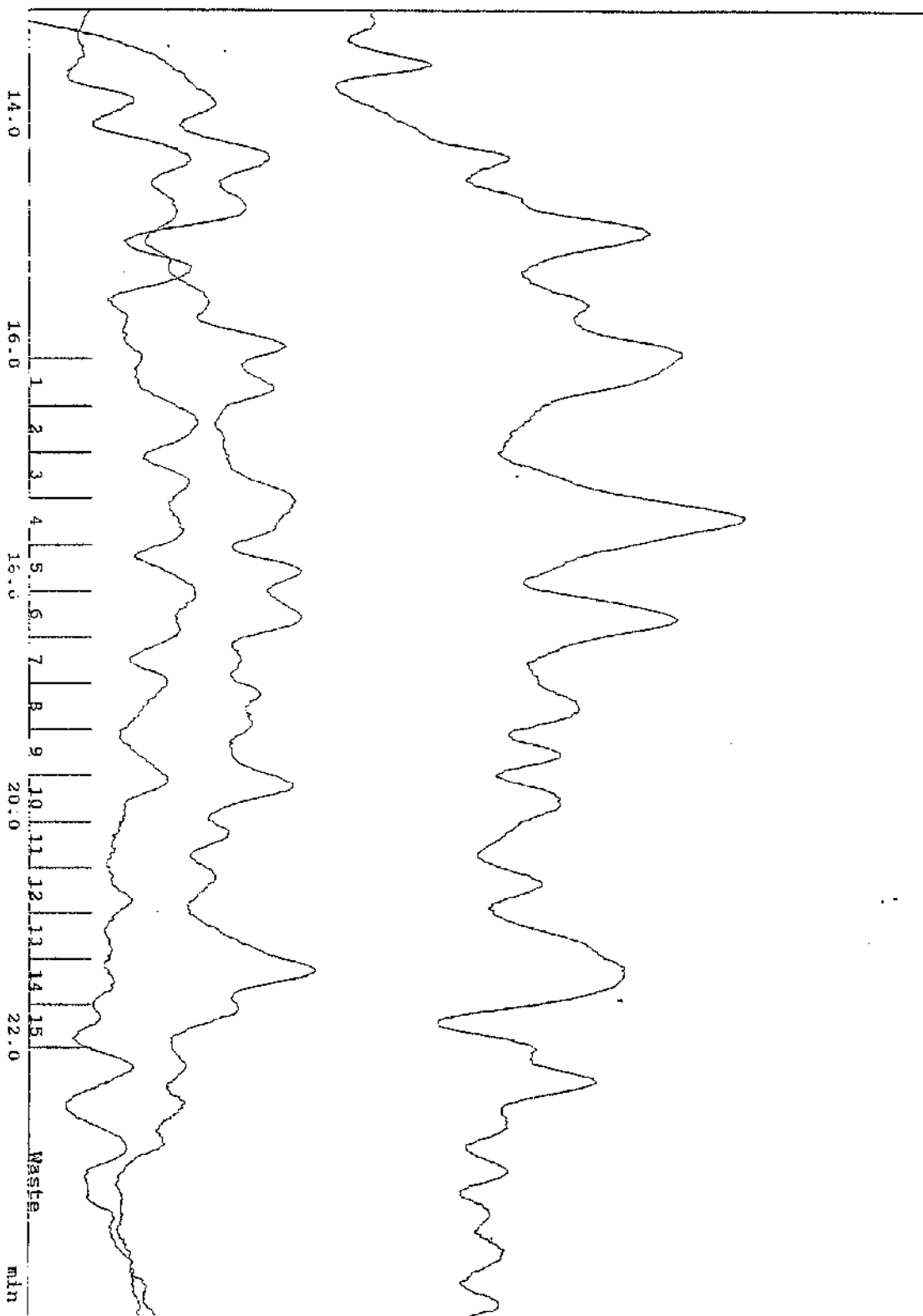
AU

0.025

0.020

0.015

0.010



min

active concentrations in the cell tracking assay. LPA was found to induce random locomotion with a speed similar to that of STMS and fMLP (table 1). This indicated that LPA was indeed having an effect on neutrophil function. Visual inspection of the cells under phase contrast microscope indicated that LPA was inducing alterations in cell morphology, with the cells polarising under the influence of LPA.

These observations led us to investigate the effect of LPA on polarisation or shape change, as well as and the fMLP and PMA-induced metabolic burst.

3.12.1) Polarisation of Human neutrophils in response to Lysophosphatidic acid

Addition of LPA to neutrophils induced very rapid shape changes and the effect was dose-dependent, in terms of the numbers of cells that became polarised. (Figs 37 a-e). Virtually 100% of cells polarised in response to 20 μ M concentration of the lipid and appeared to be similar in form to cells polarised in response to 1×10^{-6} M fMLP (Fig 37f). Higher LPA concentrations did not increase cell area, but many of the cells showed well defined spike-like projections (Fig. 37e).

Tracking analysis showed that LPA increased the speed of locomotion and gave a linear plot of the square of displacement against time, characteristic of locomotion with negligible persistence (Fig 14 e), and stimulation of locomotion by low concentrations of LPA correlated very well with the observed effects on shape change (table 2). Higher concentrations of LPA rendered the cells non-motile, but motility could be restored by the addition of albumin (table 2) indicating that the carrier solvent i.e. ethanol:chloroform was not responsible.

Table 2. The effect of different concentrations of LPA on the speed of locomotion of human neutrophils.

Treatments	Cell speed ($\mu\text{m min}^{-1}$)
PBS	2.5 ± 0.4
$0.2 \mu\text{M}$ LPA	$8.23 \pm 0.36^*$
$2 \mu\text{M}$ LPA	$11.6 \pm 0.2^*$
$20 \mu\text{M}$ LPA	$13.5 \pm 0.6^*$
$100 \mu\text{M}$ LPA	$8.2 \pm 0.18^*$
$100 \mu\text{M}$ LPA + 0.1% ALB	$16.2 \pm 2^*$
PBS + 0.1% ALB	$5.3 \pm 0.3^*$
10^{-8} M fMLP	$15.4 \pm 1.5^*$

PBS, phosphate buffered saline. LPA, lysophosphatidic acid. ALB bovine serum albumin.

Mean \pm s.e.m., n=3. $p < 0.01$ given by *

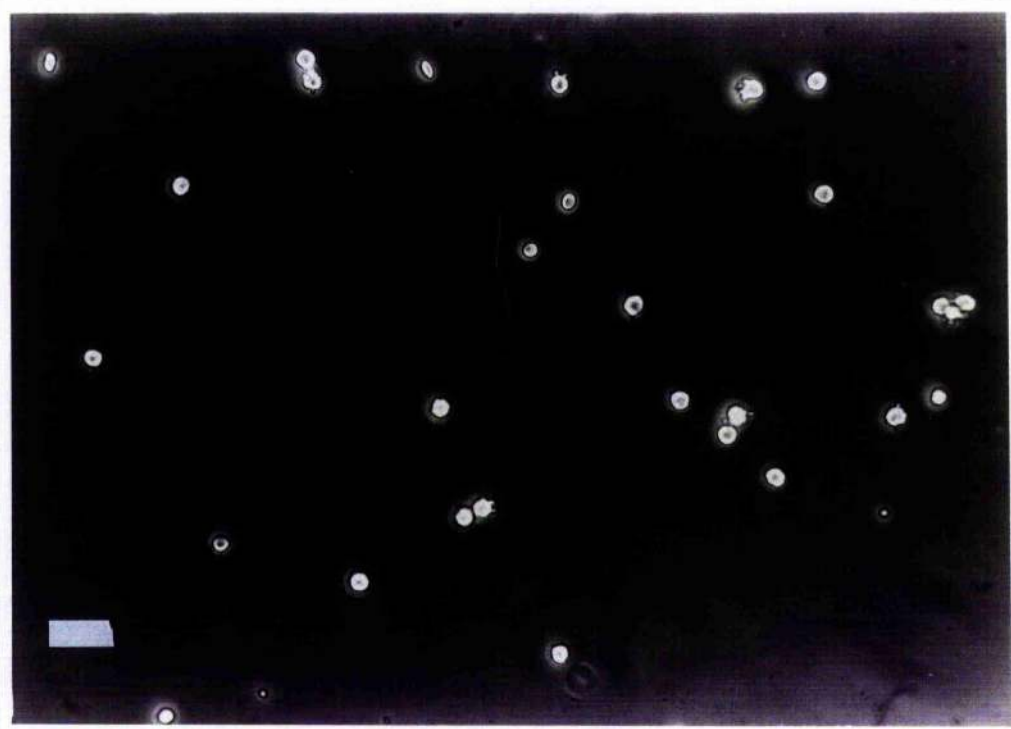
Fig. 37. The effect of lysophosphatidic acid (LPA) on the shape of human neutrophils.

Cells were fixed with 2.5% glutaraldehyde after 20 min incubation at 37°C in the presence and absence of LPA. (a) control cells in PBS, (b) cells treated with 0.2 μ M LPA, (c) cells treated with 2 μ M LPA, (d) cells treated with 20 μ M LPA, (e) cells treated with 100 μ M LPA and (f) cells treated with 10⁻⁶M fMLP.

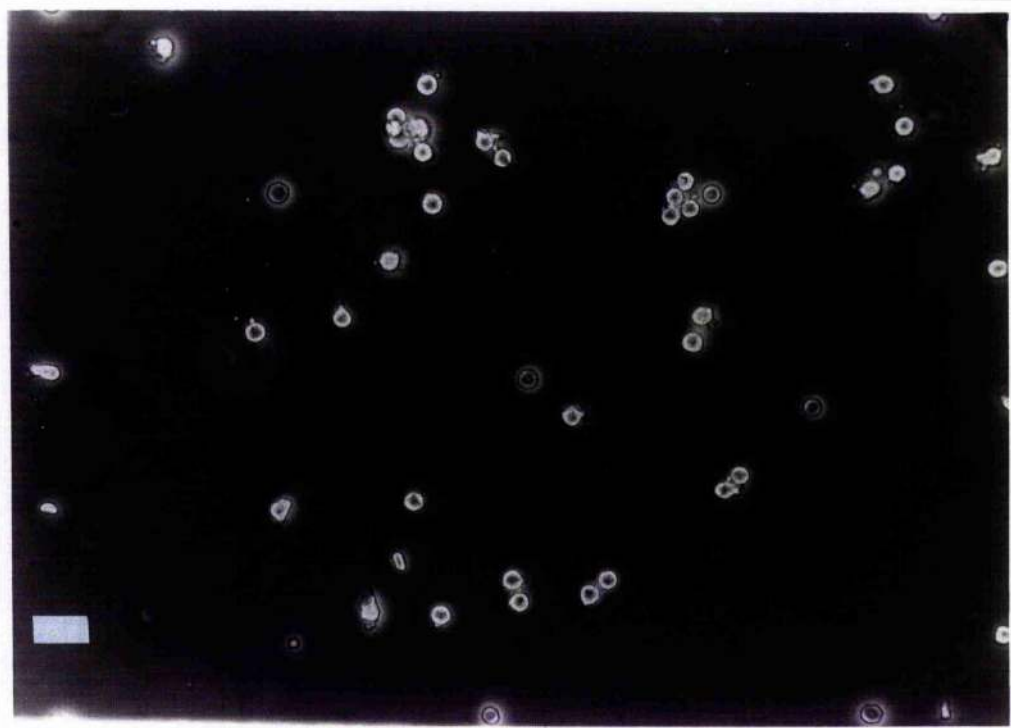
Photographs represent cell appearance under phase contrast microscopy using x32 objective.

Bars = 20 μ m.

a



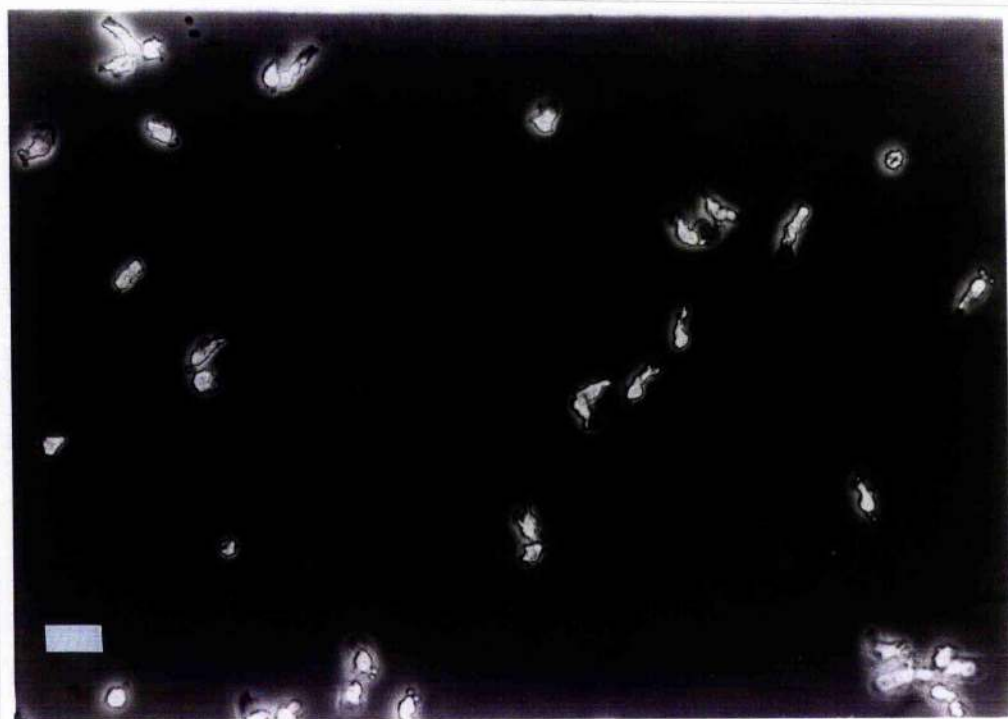
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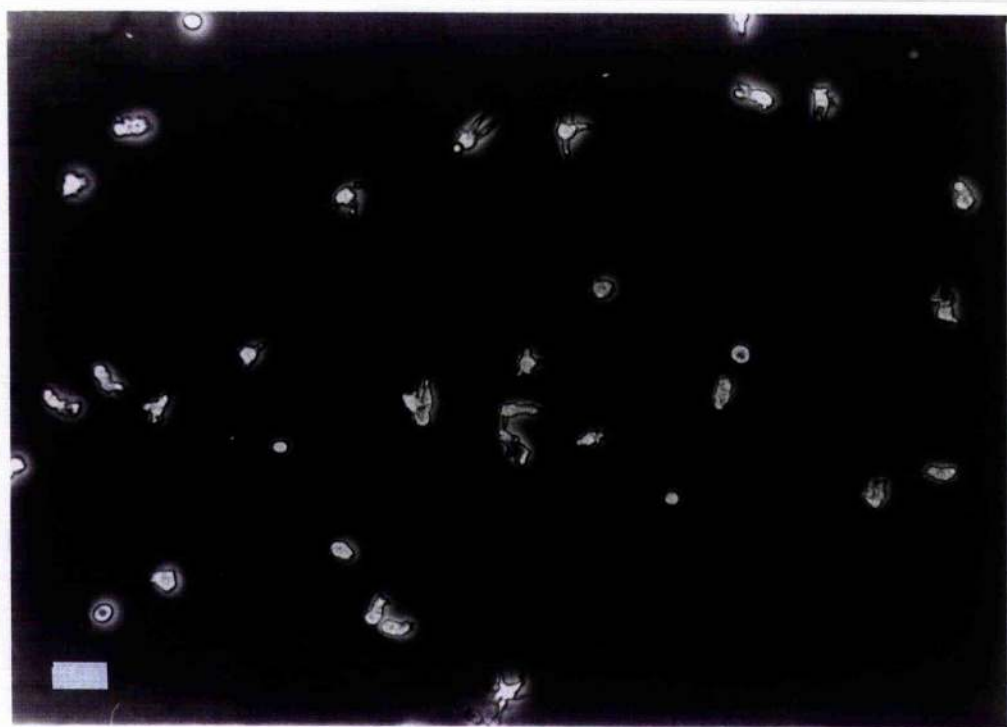
c



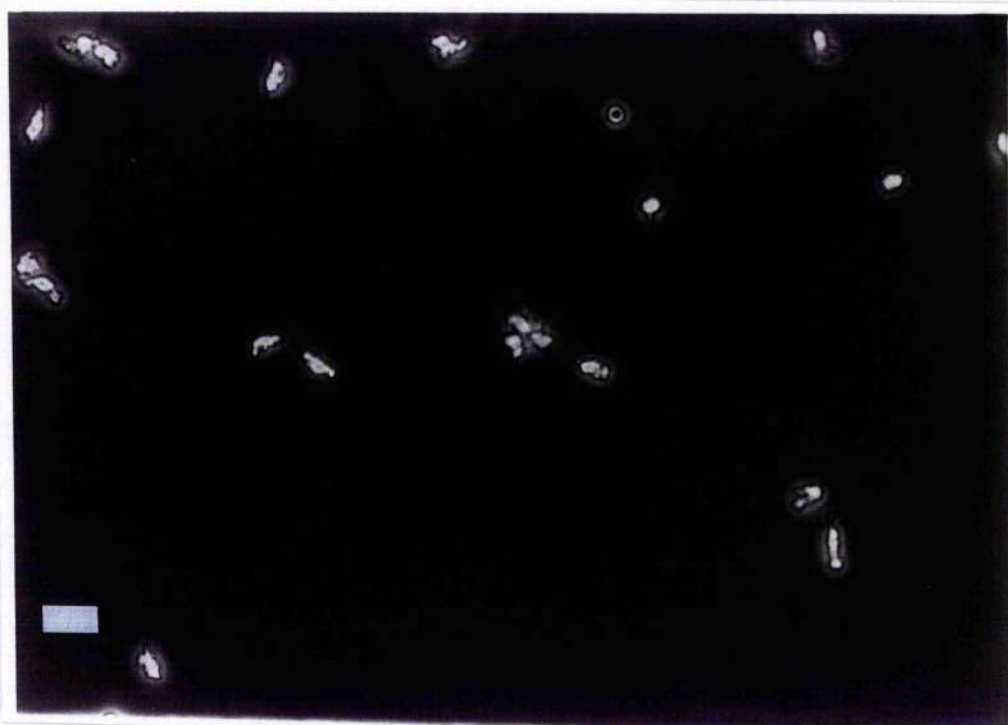
d



e



f



3.12.2.) Effect of LPA on the fMLP-induced metabolic burst of human neutrophils

The majority of agents that increase the polarisation and motility of human neutrophils can either enhance or inhibit the metabolic burst of the cells. The ability of LPA therefore both to induce the metabolic burst directly and to modulate the effect of other agents was investigated. Using DNDH-enhanced chemiluminescence, LPA was shown ineffective in stimulating the metabolic burst at concentrations below 20 μ M, although it did have a weak stimulatory effect at higher concentrations (100-200 μ M). In contrast, LPA was a powerful inhibitor of the fMLP-induced metabolic burst with a clear dose-dependent response and showed half-maximum activity at a concentration around 10 μ M. (Fig. 38). The actions of fMLP on neutrophils are known to be mediated through pertussis toxin sensitive heterotrimeric G proteins of the class G_i , and the effects of LPA in many cell types are inhibited by pertussis toxin (van Corven et al., 1993). These results indicate that the actions of both fMLP and LPA may share a common G-protein based mechanism. The results in figure 39 gave strong support for this model and showed that LPA inhibited the effect of fMLP by the same proportion when it was added as the sole inhibitor or when used together with a concentration of pertussis toxin (PTx) capable of inhibiting the response by 50%.

3.12.3.) Effect of LPA on the PMA-induced metabolic burst of human neutrophils

The results of these experiments showed two distinct effects of LPA. At low LPA concentrations there was a significant activation of the

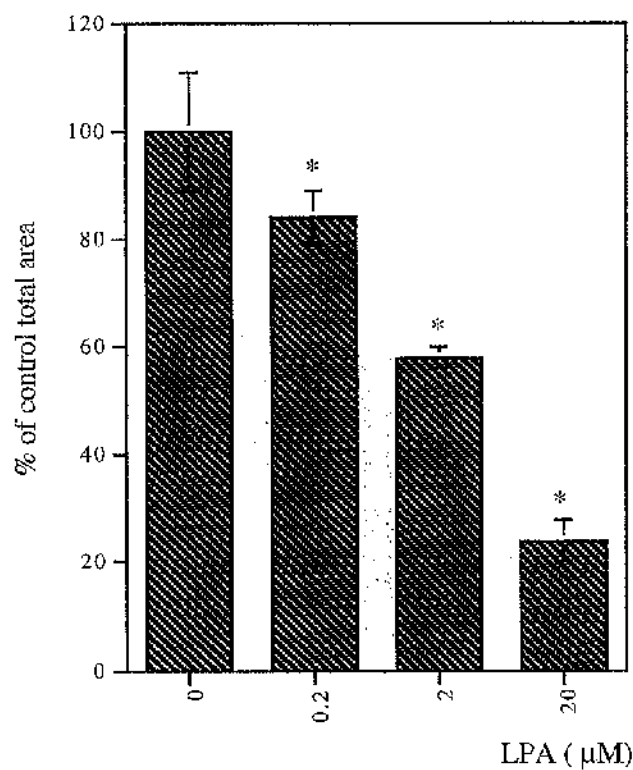


Fig. 38. The effect of different concentrations of LPA on the fMLP-induced metabolic burst of human neutrophils.

The metabolic burst was measured by DNDH-enhanced chemiluminescence, 10^{-6} M fMLP was added to 10^6 cells ml^{-1} of control and LPA-treated neutrophils.

Mean \pm S.E.M., $n=4$.

$p \leq 0.05$ given by *.

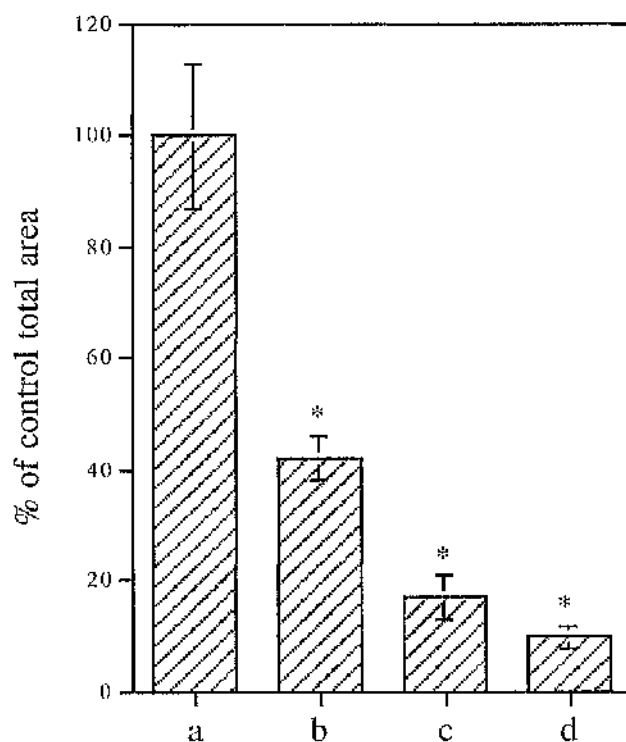


Fig. 39. The inhibitory effect of LPA on the fMLP-induced metabolic burst of pertussis toxin-treated human neutrophils.

1×10^6 cells ml^{-1} were incubated for 1 hour in the presence and absence of 100ng ml^{-1} pertussis toxin (PTx). LPA was used at a final concentration of $20 \mu\text{M}$ and fMLP at 10^{-6} M.

a). fMLP at 10^{-6} M b). fMLP + PTx. c). fMLP + LPA, and d). fMLP + PTx + LPA

Mean \pm S.E.M., $n=3$.

$p \leq 0.05$ given by *.

Results

metabolic burst induced by PMA, but at higher concentrations, LPA became a dose-dependent inhibitor of the action of PMA, causing half maximum inhibition at a concentration of around 10 μ M (Fig 40). These assays were carried out with a concentration of PMA that was sub-maximal in terms of both peak light emission and total light emission, but the degree of inhibition given by a single concentration of LPA was not very sensitive to the PMA concentration. Thus, for 10 ng/ml PMA, 20 μ M LPA gave 40% inhibition and for 1 μ g/ml PMA the same concentration of LPA gave 39.5% inhibition where the response varied two-fold, suggesting that LPA was not acting by competing directly with PMA. The time course of the inhibited response differed from lower concentrations of PMA because, although the peak time was delayed, as in lower PMA concentrations, the total duration was not altered.

3.12.4.) The effect of pertussis toxin on LPA inhibition of the PMA-stimulated metabolic burst.

To further investigate the involvement of the heterotrimeric inhibitory G protein in LPA-induced inhibition of the metabolic burst, human neutrophils were pre-incubated with a concentration of PTx which was highly effective in inhibiting the response to fMLP. This treatment had no effect whatsoever on the response to PMA (Fig. 41). When control cells and pertussis toxin-treated cells were stimulated by PMA in the presence of LPA, the response curves were virtually identical showing that the powerful inhibitory effect of LPA was neither inhibited nor enhanced by pertussis toxin, thus ruling out the involvement of pertussis toxin-sensitive G proteins in the response of the cells to PMA or the inhibition of this response by LPA.

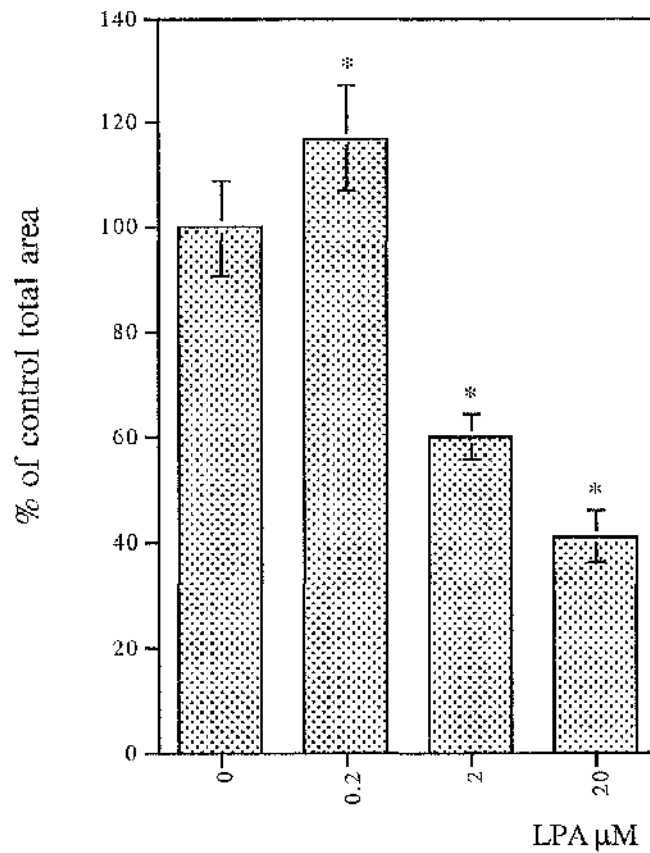


Fig. 40. The effect of different concentrations on the PMA-induced metabolic burst of human neutrophils.

The metabolic burst was measured by DNDH-enhanced chemiluminescence, 100ng ml⁻¹ PMA was added to 1 x 10⁶ cells ml⁻¹ of control and LPA-treated neutrophils.

Mean \pm S.E.M., n=4.

p \leq 0.05 given by *.

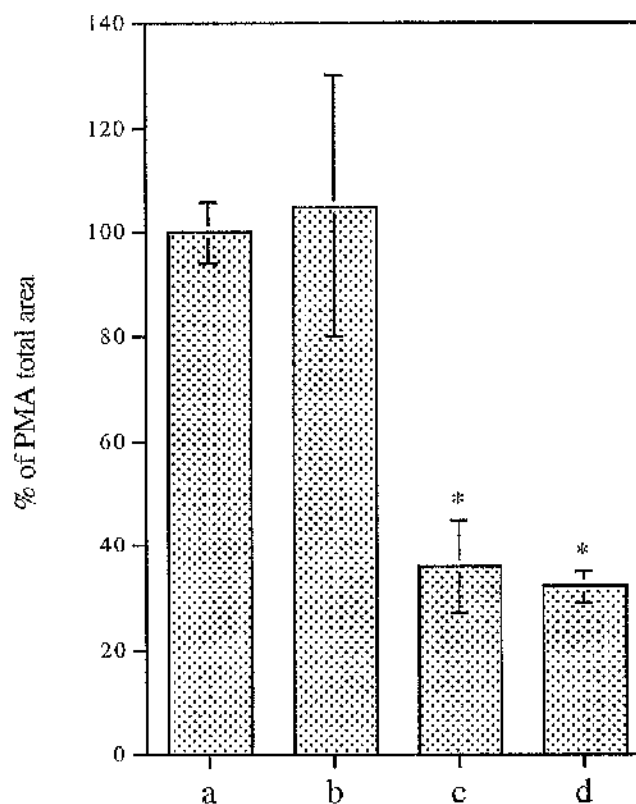


Fig. 41. The inhibition of the PMA-induced metabolic burst of pertussis toxin-treated neutrophils by LPA.

1×10^6 cells ml^{-1} were incubated for 1 hour in the presence and absence of 100ng ml^{-1} pertussis toxin (PTx). LPA was used at a final concentration of $20 \mu\text{M}$ and PMA at 100ng ml^{-1} .

a). PMA b). PMA + PTx c). PMA + LPA d). PMA + PTx + LPA

Mean \pm S.E.M., $n=3$.

$p \leq 0.05$ given by *.

3.12.5.) Effect of LPA on Degranulation of Human neutrophils

Some molecules have been shown to inhibit the metabolic burst of neutrophils simply by quenching the superoxide ions, preventing them reacting with the DNDH, and emitting light for the luminometer to detect. To rule out this false positive result for the inhibition of the metabolic burst by LPA, the fMLP-induced degranulation of neutrophils was assayed. There are a number of marker enzymes released simultaneously with the superoxide ions when neutrophils are treated with fMLP. Examples of these molecules are: myeloperoxidase, elastase, and collagenase, which can be assayed using a number of different techniques. Andrew MacLean, (unpublished result), assayed the effect of LPA on the degranulation of human neutrophils using myeloperoxidase and elastase as markers. He found that LPA significantly inhibited the fMLP-induced degranulation of human neutrophils. These results rule out any possibility of LPA quenching the DNDH-enhanced chemiluminescence results.

The primary aim of the investigations carried out in this work was to purify the active factor in the steroid-treated monocyte supernatant (STMS) responsible for dispersive motility in the automated cell tracking assay. This active molecule was also found to have other effects on neutrophil cell function, namely, alterations in F-actin distribution, cell shape, and inhibition of fMLP-induced chemotaxis. The purification of Thymosin β 4 with an oxidised methionine residue, and subsequent confirmation that it causes dispersive motility raises many interesting questions that will require further investigation.

DISCUSSION

4.1.) Dispersive locomotion of neutrophils in response to a steroid-induced factor

4.1.1.) Development of the automated cell tracking assay

The effect of steroid-induced factors on neutrophil migration is primarily of interest in elucidating anti-inflammatory mechanisms. Glucocorticoids down-regulate the synthesis of many pro-inflammatory mediators (Lew et al., 1988; Almawi et al., 1991; Standiford et al., 1992), but some of their actions can be interpreted in terms of up-regulation of anti-inflammatory mediators (Flower, 1990; Goulding et al., 1990; Wong et al., 1991). However since the initial discovery of the STMS effect, there has been some difficulty in interpreting a neutrophil migration-stimulating activity as an anti-inflammatory effect. The parameters being measured in the initial assay (Stevenson, 1978) were not clear. The contribution to the dispersion of the cell pellet, from the end of the capillary-tube, of lowered adhesion or increased cell motility were not apparent in the assay results. This led Chettibi et al., 1993 to utilise a computerised neutrophil cell tracking assay developed by Dow et al., 1987, running on a BBC Microcomputer. This assay indicated that cells treated with STMS had an increased speed of locomotion when compared with controls and IL-8, but the apparent differences in cell tracks between the samples were not being converted into differences in the persistence and diffusion coefficient.

The work described in this thesis started with the development of a procedure written in the C programming language to process the raw data from the cell tracking program written by P.D. Lawrence. The new system of neutrophil tracking was very successful in differentiating the

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obvious differences in the cell motility between different treatments that stimulated neutrophil locomotion.

The quality of locomotion induced by STMS now provides information to resolve this difficulty. Dispersive locomotion would tend to prevent cells collecting at a focus and this might become important in terminating inflammatory responses. STMS-stimulated neutrophil locomotion has a non-exploratory character, in marked contrast to that induced by fMLP where we find intense activity coupled with strong adhesion which is an ideal pre-requisite for finding junctions between endothelial cells. The type of locomotion induced by STMS coupled with, or perhaps determined by the low adhesion state of the cells is more suitable for leaving than for finding a specific site. Thus STMS activity might be one of the factors that decreases the pool of marginating neutrophils in response to steroids, and inhibits transmigration between endothelial cells.

The spectacular dispersion of cells in response to STMS presents a dramatic contrast with their response to other agents such as fMLP and IL-8 which are equally potent stimulators of the locomotor activity. However the response conforms to the pattern predicted by Gail and Boone (1970,1972) for persistent random locomotion. Their original mathematical treatment has since been extended by a number of workers. Dunn (1983) and Dunn and Brown (1987) have presented highly sophisticated analyses and have argued that motor activity at the cellular level can never be truly random. Analysis of persistent random locomotion provides a simple model in which augmented diffusion can be attributed to the single parameter, the persistence. In the present study, a population of cells is engaged in random locomotion where each cell slowly changes speed and direction and these parameters eventually become unpredictable with respect to their initial values. For this

situation, persistence is best described as the half-life ($t_{1/2}$) for randomisation of the locomotion of all of the cells with respect to their values at the t_0 , the starting time.

The surprising finding of the present work is that locomotion of cells in response to the majority of stimuli tested is not persistent. This is particularly intriguing for responses to fMLP where the results show very clear constrained diffusion which is very similar to that described by Sheetz et al., (1989) of the random movement of colloidal gold labelled cell surface proteins. Tranquillo et al., (1988) have shown that the chemotactic locomotion of neutrophils in a gradient of fMLP is persistent, but it is clear from inspection of the diffusion equations that any form of non-random locomotion must show persistence. These results also contrast with those presented by Dow et al., (1987) where neutrophils moving in response to fMLP under the same conditions presented here showed highly variable persistence.

4.2.) Tilted-stage experiments

In the modified assay, variability has been greatly reduced and the contrast between responses to fMLP, IL-8 and STMS can be attributed to differences in persistence rather than speed. The contrast in response to these factors was even more sharply defined when cell locomotion was studied in the tilt assay which was designed to increase the response to changes in cell-substrate adhesion. This makes the response to STMS highly directional and dispersion gives way to displacement as the appropriate form of analysis (Sheetz et al., 1989). The results in fig. 12 show that for all stimulants the displacement in the first 1-2 frames is high and continues at this level in the presence of STMS, but falls rapidly

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in the presence of CMS and fMLP. The sustained slope of the displacement curve is clearly a sensitive and easily measured parameter which can be obtained in the first 30 seconds of analysis, in comparison with the diffusion coefficient which relies heavily on the late data to establish the form of the curve. These results indicate that tilt probably increases the true speed of locomotion of STMS treated cells by 1.5-2 fold, but the locomotion remains that of cells moving from one point of adhesion to another, rather than free floating.

It is possible to speculate on the underlying mechanism for persistence. Electron microscope studies (Young et al., 1997, enclosed) confirm the visual observation that cells treated with STMS tend to be bipolar rather than multipolar as found with IL-8 and fMLP. The saltatory type of locomotion indicates that adhesion at one pole is broken only when a competing adhesion is established at another pole and that there is no indication of rolling locomotion as observed when the cells make initial contact with vascular endothelium (for review see Bevilacqua and Nelson, 1993) We have now shown that placing the cells in a gravitational field polarises the locomotion in response to STMS much more strongly than that induced by other stimuli. This can be interpreted in terms of the tendency of the non-adhering pole to align down-slope where highly adherent, multipolar cells would be much less affected. Again this explanation reveals a paradox. If the direction of locomotion is so readily polarised by an external force, the inherent determinant of direction must be very weak. We do not consider that there can be a significant contribution from mechanical inertia for cells moving at relatively low speeds under conditions where low Reynolds number hydrodynamics apply. It therefore seems most probable that persistence for these cells must be a consequence of bipolar shape and a natural tendency of a new adhesion to generate a molecular motor complex whose orientation is

determined by the geometry of the encounter of the cell surface with the substrate. Our results also indicate that for fMLP, one of the major mechanisms for sensing a gradient must involve differential adhesion strengths for contact sites aligned along the gradient.

4.3.) Dispersive locomotion, inhibition of chemotaxis and adhesion as the potential anti-inflammatory effect of STMS

The original assay for STMS activity, as noted at the time, did not distinguish between the consequences of reduced adhesiveness and increased motility and it is generally accepted that the steps involved in adhesion of neutrophils to endothelium are a key target for anti-inflammatory agents (Gahmberg et al., 1992; Cronstein, 1994). STMS released into the blood stream by monocytes in response to glucocorticoids would present itself to neutrophils as a uniform concentration and could therefore modify neutrophil behaviour in very much the way that we observe in our assays. Thus the cells should undergo some of the classic signs of activation, including polarisation and increased motility (Coates et al., 1992; Fernandez et al., 1995; Ehrenguber et al., 1996) however they would also undergo a dramatic decrease in adhesiveness with the expected consequence that they would not be able to adhere under conditions of even modest shear stress.

The evidence presented in this study shows that the response to STMS contrasts sharply to the effects of the pro-inflammatory, chemotactic species exemplified by IL-8, fMLP and lipid derived inflammatory mediators.

The mechanisms of neutrophil chemotaxis are still not clearly understood, but the quantitative features have been well described.

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Chemotactic locomotion of human neutrophils in a gradient of fMLP for example, is characterised by persistent locomotion up-gradient in which the cells adopt a largely bipolar morphology (Tranquillo and Moghe, 1994). This bears superficial resemblance to the behaviour induced by a uniform concentration of STMS. However the activity of the cells in a uniform concentration of chemotactic agent is characterised by very active exploratory motion, with pseudopodia forming at apparently random positions on the cell periphery and acting in conflict with one another. Each pseudopodium has high locomotory activity, but also high apparent adhesiveness to the substrate and the consequence for each cell is seen as high apparent locomotory activity as measured by the speed^{-5sec}, with very low persistence. A simple interpretation of this behaviour is that it underlies the mechanisms of gradient sensing and that pseudopodia that reach up-gradient will be reinforced by the effects of the gradient and dominate over down-gradient pseudopodia. The observations suggest that adhesive effects may be more important than pure locomotory effects in determining the outcome of the conflict.

The role of actin polymerisation in activated neutrophils has been the subject of intensive study (Coates et al., 1992). The results in this study indicate that pseudopodia are the sites of high concentrations of F-actin and the observations of motile activity indicate that this actin will be in a state of constant reorganisation. The accepted mechanisms for pseudopodium driven locomotion require some form of actin cycling between the leading edge and a juxtanuclear location behind it (Stossel, 1988). For a highly directed chemotactic response virtually all of the actin could be concentrated into this one active area. The results presented here and in Young et al., 1997, show no such extreme bipolarity in response to uniform concentrations of chemotactic agents on protein coated glass and neither is the morphology in cells treated, and

then fixed, in suspension. However, STMS-treated neutrophils are highly bipolar and furthermore appear to have a major concentration of F-actin at one pole and a secondary, minor concentration at the opposite pole. These cells do not have the overall degree of activity of cells responding to a chemotactic agent, but appear to be more highly motile because the locomotion is more persistent. The mechanism of locomotion is obscure because the cells appear to be weakly tethered to the glass by one 'tail' whilst the other moves freely until the cell appears to attach to, and move to, a distant site. The obscurity lies in the fact that because the cell appears to have a single site of adhesion, and is freely motile, there is no obvious reason why movement should continue in the previously determined direction. It is also not clear what role actin may play in this type of locomotion. There are two extreme models; the looping model and the tumbling model. In the looping model the tail of the cell would be the major point of adhesion whilst the head would make exploratory movements and then adhere, causing the tail to detach and move close to the head. In the tumbling model each end would act in turn as an adhesion point and the corollary is that the polymerised actin would have to pass from one pole of the cell to the other. Here the speed of locomotion would be governed by the rate at which loss of actin from the trailing pole allowed the leading adhesion to dominate over the trailing adhesion. In this model the directed flow of actin to the distant pole would underlie directional persistence.

The effect of STMS on chemotactic responses also sheds considerable light on its possible mode of action. STMS is not itself a chemotactic agent, but is a powerful inhibitor of the chemotactic response to fMLP. It appears to short-circuit chemotactic responses by inducing a highly polarised structure without any aspect of gradient sensing, but unlike other polarising stimuli, it causes a dramatic decrease in

adhesiveness to protein coated-glass (Chettibi et al., 1993), bovine aorta endothelial cells, and Human umbilical endothelial cells (Young et al., 1997, enclosed). Observations of the morphology of the cells and of the internal organisation give some indication why this should be. The surface of STMS-treated neutrophils, seen in scanning EM is readily distinguished from that induced by chemotactic agents by its extreme smoothness (Young et al., 1997, enclosed). There is evidence that some forms of adhesion, for example that mediated by L-selectin is greatly increased by the clustering of the lectin on microvilli (von Andrian et al., 1995). Following this example we propose that neutrophil adhesion may depend on the presence of surface projections, the lack of which in STMS-treated cells could explain the lowered levels of adhesion observed.

4.4.) Thymosin β 4 as an anti-inflammatory mediator of Glucocorticoids

4.4.1.) Oxidation as a modulator of Inflammation

Methionine is the most readily oxidised amino acid, and a number of peptides and proteins have been described whose conformation, and or, function is altered by the oxidation of one or more methionine residues. Examples of altered conformation caused by methionine oxidation are: interferon α -2B, where a single methionine oxidation altered conformation, and immuno-reactivity to a monoclonal antibody, although no change in function was observed (Gitlin et al., 1996). There is reduced activity in human parathyroid hormone following oxidation of one of two, and both methionine residues (Nabuchi et al., 1995).

The oxidative state of a number of molecules has been implicated in inflammation, as a signal of cellular damage. Oxidation of methionine residues inactivates bacterially derived chemotactic peptides like fMLP, whilst oxidation of pro-inflammatory lipid mediators gives rise to a broad spectrum of products, some with pro-inflammatory and some with anti-inflammatory effects (Jira et al., 1997; Girona et al., 1997). Oxidation of a single methionine residue inactivates $\alpha 1$ anti-trypsin (the major inhibitor of neutrophil elastase) (Hall and Roberts, 1992; Cox and Billingsley, 1984) and the significance of this is underlined by the poor prognosis for insult induced lung damage for individuals with low $\alpha 1$ anti-trypsin levels. The oxidation of the methionine in thymosin $\beta 4$ has also been shown to reduce its actin binding in *in vitro* studies (Huff et al., 1995).

The oxidation state of host inflammatory mediators may be a useful signal to leucocytes that the inflammatory response is causing damage to host tissue and reduce the avidity of that response. The oxidation of a uniquely eukaryotic peptide, Thymosin $\beta 4$, makes it an ideal candidate as a signal to reactive oxygen producing phagocytic cells that the inflammatory response is causing host cell damage, and the inflammatory response needs to be reduced.

4.4.2.) Potential anti-inflammatory mechanisms of T $\beta 4$ ox

The finding that Thymosin $\beta 4$ with an oxidised methionine residue (T $\beta 4$ ox) is produced by monocytes cultured in the presence of anti-inflammatory glucocorticoids and is responsible for the dispersive locomotion induced by STMS implies that it may have a role as a

mediator of the anti-inflammatory effects of glucocorticoids. A number of potentially anti-inflammatory functions have so far been ascribed to T β 4ox, in this study and also by Andrew MacLean, 1997.

4.4.2.1.) Dispersive locomotion

The type of locomotion induced by T β 4ox coupled with, or perhaps determined by the low adhesion state of the cells is a potential anti-inflammatory mechanism, in that, persistence of the cells in a uniform concentration of agonist is more suitable for leaving than for finding a specific site. The release of T β 4ox at a site of inflammation by macrophages could have the effect of causing the neutrophil to leave the immediate area of inflammation. This can best be described in terms of the original assay for STMS by Stevenson, 1978, the capillary-tube migration assay (Fig. 5), in which neutrophils were seen to migrate away from the end of the capillary-tube in response to STMS. This type of behaviour is mimicked, to some degree, by the response of neutrophils in the cell tracking assay track displays (Fig 13), where the normalised cell tracks for STMS disperse away from the centre of the axes, whilst the tracks for CMS, and other neutrophil motility activating factors remain close to the centre due to the low persistence of the cells.

4.4.2.2.) Adhesion

The results presented in this study show the inhibition, by STMS of adhesion of human neutrophils to bovine aorta endothelial cells (Fig. 18). Previous work by Chettibi et al., 1993, showed that STMS inhibited the

adhesion of neutrophils to bovine serum albumin-coated coverslips, and further work has been carried out by MacLean, 1997, in this laboratory, showing the same inhibitory effect of STMS, and HPLC purified active samples, using human umbilical vein endothelial cells (HUVEC) activated with IL-1 as the adhesive substrate. The adhesion of leucocytes to vascular endothelium is a crucial stage in their recruitment to an inflammatory site, and any disruption to this process has dramatic effects on the ability of leucocytes to combat infectious agents, seen in leucocyte adhesion deficiency (LAD) (Anderson and Springer, 1987). However, in inflammatory disorders where there is excessive leucocyte infiltration into tissue without apparent insult, a mediator that reduces adhesion of leucocytes to the endothelium would be of great therapeutic benefit. The mechanism by which glucocorticoids reduce the number of marginating neutrophils is thought to be by inhibition of their adhesion to vascular endothelium. Part of this reduction of adhesion is probably due to the decrease in the surface expression of endothelial adhesion molecules ICAM-1 and L-selectin on endothelial cells (Cronstein et al., 1992). Release of T β 4ox by blood monocytes in patients under glucocorticoid therapy could also account for a decrease in the number of marginating neutrophils.

4.4.2.3.) Chemotaxis

The mechanism by which neutrophils are able to detect and move up a chemotactic gradient is unknown. There are two main models proposed, and a third is a hybrid of the first two. The temporal model involves the cells being able to remember the concentration of agonist at a particular time, move to another site, and compare the levels, deciding to move

towards the higher concentration. The mechanism by which a cell 'remembers' is unknown. The spatial model proposes that a cell is able to detect a small change in concentration across the length of its cell by differences in the number of agonist receptors occupied, choosing to move in the direction with more occupied receptors. However the neutrophil detects a chemotactic gradient and moves towards a site of inflammation, it is a fundamental function of the cell *in vivo*, and inhibiting this would be potentially of great therapeutic benefit. STMS starting material is a potent inhibitor of the fMLP-induced chemotaxis of neutrophils in the modified Boyden chamber assay (section 3.6). It remains to be tested if T β 4ox is an inhibitor of this particular function of neutrophils.

4.4.2.4.) Degranulation

Although no inhibitory effect on the fMLP or PMA-induced release of superoxide ions by human neutrophils has been found, using partially-purified HPLC samples or neutrophil buffy coat T β 4ox (results not shown), MacLean, 1997, has found a significant inhibitory effect of partially-purified HPLC samples, active in our cell tracking assays, on the fMLP-induced degranulation of human neutrophils. It remains to be seen if T β 4ox present in these active samples was the mediator responsible for this inhibitory effect, but would be consistent with its potential anti-inflammatory role, preventing the release of enzymes, such as, elastase and collagenase.

4.5.) Parallel purification of T β 4ox from STMS and CMS

The results of parallel purification of thymosin β 4 from STMS and CMS (section 3.11) show that there is more T β 4ox present in STMS than CMS, and indeed the UV trace for CMS at the elution point of T β 4ox is indistinguishable from the baseline trace. This would appear to confirm the glucocorticoid induction of secretion of T β 4 by steroid-treated monocytes. It is not clear whether T β 4 is actively secreted by the monocytes, or released by cell damage caused by the glucocorticoid presence. Stevenson, 1978, however, showed that at the concentration of steroid being used in the culture of monocytes used here, no significant cell damage occurred. This would imply that the cells are actively secreting T β 4ox into the culture medium.

The result of the parallel purification of STMS and CMS does not give a clear cut answer to the question of the relative quantity of T β 4ox being released by the cells under both culture conditions as the signal to noise ratio in the samples is low, and will therefore require a repeat of this procedure using a greater starting volume for each.

4.6.) Oxidation of Thymosin and its release by glucocorticoid treated monocytes

The mechanism by which the monocyte could oxidise and release thymosin β 4 is unclear. The vast majority of secreted proteins leave the cell by the classic ER/golgi/ route and they pass to the PM by either the constitutive or the regulated secretion pathways. An increasing number apparent exceptions are now being identified where the polypeptide does not possess an ER translocation signal. These polypeptides appear to be

translated on cytoplasmic ribosomes and then pass directly through the PM by an unspecified mechanism. Examples of this are lipocortin 1, a cytosolic protein which also has an extracellular pool and has been implicated in modification of neutrophil behaviour. This polypeptide lacks an ER signal yet is efficiently exported from monocytes / macrophages (Comera and Russomarie, 1995). Interestingly, although there is a large cytosolic pool of lipocortin 1, and the synthesis appears to be steroid regulated, the export also appears to be steroid regulated (Comera and Russo-marie, 1995; Philip et al., 1997; Mizuno et al., 1997). This molecule shares another feature with thymosin β 4 in that it has an N-terminal acetyl group. The hypothesis has been proposed that proteins which have this post-translational modification are by and large those with a long residence time in the cytosol; the implication being that the acetylase enzyme is non-specific and slow acting.

IL-1 is also exported directly through the PM, although in this case the export appears to be coupled with proteolytic cleavage catalysed by IL-1 converting enzyme (ICE).

From this it can be concluded that a number of proteins which could be involved in signalling can be secreted directly from the cytosol. Furthermore a number of these show the characteristic feature associated with long residence time in the cytosol. This sub-class also share the interesting feature in that all have been proposed to interact with actin. The secretion of lipocortin-1 by a steroid dependent mechanism and the acetylated N-terminal shows apparent similarities to the release of T β 4ox by steroid-treated monocytes.

There are two possible sites at which T β 4ox could act as an anti-inflammatory signal.

Release of T β 4 in its native state by monocytes at an inflammatory site and subsequent oxidation by reactive oxygen species produced by neutrophils could signal these cells, via a specific receptor for the oxidised derivative, to 'switch off' their inflammatory responses and leave the site by the dispersion mechanism described above. Diffusion of T β 4ox from the site could then affect the adhesion of marginating, and chemotaxis of migrating neutrophils, preventing them from successfully reaching the inflammatory site.

The second site of action is in the bloodstream. Release of T β 4ox by blood monocytes could reduce the number of vascular endothelium-adherent neutrophils, as seen in patients under glucocorticoid therapy.

4.7.) Lysophosphatidic acid: An induces neutrophil locomotion and inhibits their fMLP and PMA-induced metabolic burst

The study into the effects of LPA as a mediator of neutrophil function came from our initial neutrophil tracking assays, in which we compared the motility induced by a number of neutrophil locomotion stimulators. LPA has been previously reported to have no effect on intracellular calcium concentration in human neutrophils (Jalink et al., 1990), but little work had been carried out on its effect on other neutrophil functions and responses, and since LPA is released at physiological concentrations by thrombin-activated platelets, 1-1.5 μ M (i.e. 0.5-2.5 μ g/ml) (Eichholtz et al., 1993), it seemed reasonable that it would have some effect on immune cells infiltrating into a wound site.

In the present studies, LPA affected neutrophil shape change and activated motility. In addition this lipid was a powerful inhibitor of the

production of oxygen species (the metabolic burst) by human neutrophils in response to fMLP and PMA.

4.7.1.) Intracellular mechanism of LPA effects on human neutrophils

LPA has been reported to affect various cell functions through the activation of a family of membrane-bound G proteins, some of which are pertussis toxin-sensitive and some pertussis toxin insensitive (van Corven et al., 1993). The results in this study show an apparently simple mechanism for the action of fMLP consistent with the involvement of heterotrimeric inhibitory G protein (G_i). Thus LPA antagonises the effect of fMLP by lowering the activation of G_i whilst pertussis toxin achieves the same effect by the ADP-ribosylation of $G_{i\alpha}$ subunit (Kumagia et al., 1993). When the two agents were used in conjunction, the residual activity present when the action of fMLP was partially inhibited by LPA and further reduced by the presence of pertussis toxin was consistent with the action of a common mechanism.

An obvious consideration when interpreting these results is to consider the possibility that LPA is in some way reducing the detected metabolic burst by a quenching mechanism as seen with some other molecules tested in a chemiluminescence assay. To rule this out as a possibility the fMLP-induced degranulation of human neutrophils was assayed and similar inhibition was observed by LPA (Dr. A.G. MacLean, personal communication). These types of assay are not open to the same artefact possibilities as chemiluminescence, so it is safe to conclude from these results that the observed inhibition of the metabolic burst of human neutrophils by LPA was through a cellular process.

Since both LPA and PTx alone can produce total inhibition of the fMLP-mediated response, it is difficult to design experiments in which alternative pathways of inhibition could be identified. In contrast the inhibition of PMA-simulated metabolic burst by LPA shows an effect that cannot be attributed to the same heterotrimeric inhibitory G proteins because the action of PMA occurs at a later point in the pathway and LPA-induced inhibition of the PMA-stimulated metabolic burst in both pertussis toxin-treated and untreated cells was identical.

An alternative explanation follows very readily from consideration of the involvement of the small cytosolic GTP-binding proteins in the regulation mechanism of NADPH-oxidase activity. Recently a number of cytosolic GTP-binding proteins (G_{ox}) have been reported to be involved in the regulation of the metabolic burst enzyme, NADPH-oxidase (review see Morel et al., 1991). These include, *Rac 2* a member of the *Ras* super family of GTP-binding proteins (Knaus et al., 1991), *p67phox* and *p47phox*, which were identified by their absence in patients with chronic granulomatous disease and the $\phi 1$ which was identified as the small GTP-binding protein *p21rac1* and the GDP-dissociation inhibitor *rhoGDI* (Abo et al., 1991). *In vivo* experiments showed that LPA was among the phospholipids able to inhibit rhoGAP (Tsai et al., 1989) and recent work has shown that LPA was able to stimulate the rho family of small GTP-binding proteins that are involved in the regulation of actin stress fibres and the assembly of focal adhesions in fibroblasts (Ridley and Hall, 1992; Ridley et al., 1992).

Although the regulation of small GTP-binding proteins by LPA in human neutrophils has not been reported elsewhere, the present results provide good evidence for the action of LPA via the small GTP-binding protein. The mechanism of the effect of LPA on neutrophil shape change,

locomotion and inhibition of the metabolic burst in this study could be mediated through its effects on these small GTP-binding proteins. Clearly any action at the penultimate stage in the signal transduction pathway obscures any upstream effects and it is therefore not clear if LPA inhibits the action of fMLP by acting both at an early stage to block the trimeric G proteins and at a late stage to block the action of the small GTP-binding protein. The alternative model is that the action of LPA on the same GTP-binding protein is modulated via the trimeric GTP-binding protein. Obviously, further experiments are required to address the direct involvement of these small cytosolic GTP-binding proteins in the regulation of the neutrophil activation and their regulation by LPA.

4.7.2.) Role of LPA as an inflammatory mediator

These results imply that LPA could have a modulatory effect on neutrophils following an injury. Released by platelets during blood clotting, its proliferative effects on a number of cell types, including fibroblasts, could aid in tissue repair. It will be of interest to see if LPA exerts any chemotactic stimulus on fibroblasts encouraging them to migrate towards the damaged site and speed up the repair of tissue. The stimulatory effect of LPA on the migration of neutrophils, in this study, does not have an obvious function *in vivo*. It remains to be seen if LPA is chemotactic for neutrophils *in vitro*, which could provide a role in attracting the cells to a site of possible infection. The inhibitory effect of LPA on degranulation and the metabolic burst seems to conflict with this role, in that, a factor with a pro-inflammatory function to activate the motility and attract neutrophils from the bloodstream then inhibits their main anti-microbial functions. The possible role that LPA has in inflammation remains to be clarified by further investigation.

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APPENDIX

Program written in C, to run on an Acorn Archimedes microcomputer; to display results from tracking assay

Calculates average cell displacement, speed, drift and direction of drift of cell population.

Displays graphs of: Average speed vs time, Displacement vs time, and displacement squared vs time.

Allow the user to draw a line, using the mouse control, that corresponds to the linear part of the displacement squared graph; outputting the parameters of cell locomotion, namely, diffusion coefficient and persistence.

```
#include "track.h"
#include <bbc.h>
#include <sprite.h>
#include <time.h>
#include <math.h>
extern celldata cellarray[FRAMES][MAXCELLS];
extern int interval;
FILE *file;

void cell_pos_speed( int frame,char *name, int numactive )
{
    int fr, cell;
    int no_readings;
    int total_no_readings = 0;
    int x_start = 48;
    int y_start = 388;
    int x_increment = 428;
    int x_start1 = x_start + x_increment;
    int x_start2 = x_start + 2 * x_increment;
    double sp_dr_x = 0;
    double sp_dr_y = 0;
    double drift_speed = 0;
    double sp_drift_x = 0;
    double sp_drift_y = 0;
    double x_drift = 0, y_drift = 0;
    double drift_angle = 0;
    double x_dr = 0;
    double y_dr = 0;
    double drift = 0;
    double tot_drift = 0;
    double mean_x_dr = 0;
    double mean_y_dr = 0;
    int x_step;
    int x,y,buttons;
    double x0, y0, x1, y1;
    double diffusion;
    double paths = 0;
    double sum_xvals = 0, sum_yvals = 0;
    double total_sum_speed = 0;
    double frame_mean = 0;
    double d_paths;
    double dy_val = 0;
    double sum_speed = 0;
    double mean = 0;
    double y_val = 0;
    double init_area = 0;
    double final_area = 0;
```


Appendix I

```
double sum_area = 0;

int d2y_co = 0;
int dd2y_co = 0;
int d2time = 0;
int dy_co = 0;
int ddy_co = 0;
char path [80];

bbc_gcol ( 0, 7 );
bbc_move( x_start, y_start);
bbc_draw( x_start + 400, y_start );
bbc_move( x_start, y_start);
bbc_draw( x_start, y_start + 600 );
bbc_move( x_start, y_start + 300 );
bbc_draw( x_start + 10, y_start + 300);
bbc_move( x_start, y_start + 600 );
bbc_draw( x_start + 10, y_start + 600 );
bbc_move( x_start + 200, y_start);
bbc_draw( x_start + 200, y_start + 10);
bbc_move( x_start + 400, y_start);
bbc_draw( x_start + 400, y_start + 10);
bbc_move( x_start1, y_start);
bbc_draw( x_start1 + 400, y_start );
bbc_move( x_start1, y_start);
bbc_draw( x_start1, y_start + 400 );
bbc_move( x_start1, y_start + 200 );
bbc_draw( x_start1 + 10, y_start + 200);
bbc_move( x_start1 + 200, y_start );
bbc_draw( x_start1 + 200, y_start + 10 );
bbc_move( x_start1, y_start + 400 );
bbc_draw( x_start1 + 10, y_start + 400 );
bbc_move( x_start2, y_start);
bbc_draw( x_start2 + 400, y_start );
bbc_move( x_start2, y_start);
bbc_draw( x_start2, y_start + 400 );
bbc_move( x_start2, y_start + 200 );
bbc_draw( x_start2 + 10, y_start + 200);
bbc_move( x_start2 + 200, y_start);
bbc_draw( x_start2, y_start + 400 );
bbc_draw( x_start2 + 10, y_start + 400);
paths = 0; no_readings = 0; x_drift = 0; y_drift = 0;
for( cell = 0; cell < numactive; cell++ )
{
    if( cellarray[frame - 1][cell].valid )
    {
        no_readings++;
        paths += (double)0.36 * (double)sqrt( fsqr( (double)cellarray[frame - 1][cell].centre.x -
            (double)cellarray[0][cell].centre.x) + (double)fsqr(cellarray[frame - 1][cell].centre.y
            - (double)cellarray[0][cell].centre.y) );
    }
}
diffusion = sqrt(paths / no_readings);
strcpy( path, "$comma." );
strcat( path, name );
file = fopen( path, "w" );
for( fr = 1; fr < frame; fr++ )
{
    no_readings = 0; sum_xvals = 0; sum_yvals = 0; sp_drift_x = 0; sp_drift_y = 0; sp_dr_x = 0;
    sp_dr_y = 0;
    for( cell = 0; cell < numactive; cell++ )
```

```
if( cellarray[fr][cell].valid )
{
    no_readings++;
    sum_xvals += ((double)cellarray[fr][cell].centre.x - (double)cellarray[0][cell].centre.x);
    sum_yvals += ((double)cellarray[fr][cell].centre.y - (double)cellarray[0][cell].centre.y);
    x_drift = sum_xvals/no_readings;
    y_drift = sum_yvals/no_readings;
    sp_drift_x += ((double)cellarray[fr][cell].centre.x - (double)cellarray[fr-1][cell].centre.x);
    sp_drift_y += ((double)cellarray[fr][cell].centre.y - (double)cellarray[fr-1][cell].centre.y);
}
sp_dr_x = sp_drift_x/no_readings;
sp_dr_y = sp_drift_y/no_readings;
drift_speed = (double)sqrt(fsqr(sp_dr_x) + (double)fsqr(sp_dr_y)) ;
tot_drift = ((double)sqrt(fsqr(x_drift) + (double)fsqr(y_drift))) * 0.36;
x_dr += x_drift;
y_dr += y_drift;
sum_speed = 0, paths = 0, d_paths = 0; no_readings = 0; sum_area = 0;
for( cell = 0; cell < numactive; cell++ )
if( cellarray[fr][cell].valid )
{
    no_readings++;
    sum_speed += (( sqrt( fsqr (( (double)cellarray[fr][cell].centre.x
        - (double)cellarray[fr - 1][cell].centre.x))
        + (double)fsqr( cellarray[fr][cell].centre.y
        - (double)cellarray[fr - 1][cell].centre.y)))
        * 4.32 ) - (double)(drift_speed * 4.32);
    /* convert pixels per 5 seconds to microns per minute */
    paths += ( 0.36 * (double)sqrt( fsqr( cellarray[fr][cell].centre.x
        - (double)cellarray[0][cell].centre.x )
        + (double)fsqr( cellarray[fr][cell].centre.y
        - (double)cellarray[0][cell].centre.y )))
        - tot_drift;
    d_paths += 0.36 * (double)sqrt( fsqr( cellarray[fr][cell].centre.x
        - (double)cellarray[0][cell].centre.x )
        + (double)fsqr( cellarray[fr][cell].centre.y
        - (double)cellarray[0][cell].centre.y ));
    sum_area += ( cellarray[fr][cell].area);
}
if(fr == 1) init_area = 0.36 * 0.36 * (sum_area/no_readings);
if(fr == frame - 1) final_area = 0.36 * 0.36 * ( sum_area/no_readings);
total_no_readings += no_readings;
total_sum_speed += sum_speed;
frame_mean = sum_speed / no_readings;
mean = (total_sum_speed / total_no_readings);
y_val = fsqr(paths / no_readings);
dy_val = fsqr(d_paths / no_readings);
x_step = (2 * interval * fr/(frame/40 + 1));
bbc_gcol(0, 7);
bbc_circle(x_start + x_step, y_start + 2.8 * y_val, 6);
bbc_gcol(3, 1);
bbc_rectangle(x_start + x_step, y_start + 2.8 * dy_val, 8,8);
bbc_gcol(0, 2);
bbc_circle( x_start2 + x_step, y_start + 4 * no_readings,6);
bbc_gcol(0, 6);
bbc_circle( x_start1 + x_step, y_start + 20 * paths / no_readings,6);
bbc_gcol(0, 3);
bbc_rectangle( x_start1 + x_step, y_start + 20 * d_paths / no_readings ,8,8);
bbc_gcol(0, 5);
bbc_circle( x_start1 + x_step, y_start + 20 * tot_drift ,6);
bbc_gcol(0, 3);
bbc_circle( x_start2 + x_step, y_start + 15 * frame_mean,6 );
```

Appendix I

```
/* comma separated file */
d2y_co = y_val;
dd2y_co = dy_val;
d2time = interval * fr;
dy_co = 10 * paths / no_readings ;
ddy_co = 10 * d_paths / no_readings ;
fprintf(file, "%d", d2time );
fprintf(file, "%4.2f", (float)dy_co/10 );
fprintf(file, "%4.2f\n", (float)ddy_co/10 );
/*fprintf(file, "%d", d2time );
fprintf(file, "%d", d2y_co );
fprintf(file, "%d", dd2y_co );
fprintf(file, "%d\n", d2time );*/
fclose( file );
mean_x_dr = x_dr/frame;
mean_y_dr = y_dr/frame;
drift=(double)( sqrt(fsqr(x_dr * 0.36) + (double)fsqr(y_dr * 0.36) ))/frame;
drift_angle = atan2 (mean_y_dr , mean_x_dr );
text_colour(2);
tab( 4, 23); printf( "Diff. coeft" );
tab( 4, 24); printf( "Persistance" );
tab( 57, 28); printf( "Frames");
text_colour( 2 );
tab( 57, 24); printf( "Cells");
text_colour( 2 );
tab( 57, 27); printf( "Area");
text_colour(2);
tab( 57, 23); printf( "Speed");
text_colour(2);
tab( 32, 24); printf( "Drift" );
text_colour(2);
tab( 32, 23); printf( "Distance");
text_colour(7);
tab(17, 23 );
printf("??? ");
tab(17, 24 );
printf("??? ");
/* print mean speed corrected for jitter of 2-3 in y-axis */
text_colour(3);
tab(65, 23); printf( "%3.1f", (mean ) );
printf( "µm/min");
text_colour(7);
tab(4, 1); printf( "200µm≤");
text_colour(2);
tab(65, 24);
printf( "%d-%d ", numactive, no_readings);
text_colour(7);
tab(65, 27);
printf( "%3.1f", init_area);
printf( "µm≤");
printf( "%3.1f", final_area );
printf( "µm≤");
text_colour(5);
tab( 42, 24);
printf( "%2.2f", drift);
printf( "µm at ");
printf( "%1.0f", (drift_angle * 360)/6.28);
printf( "∞");
text_colour(6);
tab( 42, 23); printf( "%4.2f", paths/no_readings );
printf( "µm" );
```

Appendix I

```
pause();
mouse( &x, &y, &buttons );
x0 = x; y0 = y;
mouse( &x, &y, &buttons );
bbc_move(x0, y0);
bbc_draw( x, y );
x1 = x; y1 = y;
do
{
mouse( &x, &y, &buttons );
if( x != x1 || y != y1 )
{
bbc_gcol(3,6);
bbc_move(x0, y0);
bbc_draw( x1, y1 );
bbc_move(x0, y0);
bbc_draw( x, y );
x1 = x; y1 = y;
if( x0 != x1 )
{
text_colour(6);
tab( 17, 23); printf( "          "); tab( 17, 23);
printf( "%2.3f", (y0 - y1)/( 4 * (x0 - x1)));
printf( "µm≤/sec.");
}
if(y0 != y1 )
{
tab( 17, 24); printf( "          "); tab(17, 24 );
if(frame > 40)
{
printf( "%4.2f", (x0 - x_start - (x0 - x1) * (y0 - y_start)/(y0 - y1)));
printf( "sec.");
}
else
{
printf( "%4.2f", (x0 - x_start - (x0 - x1) * (y0 - y_start)/(y0 - y1))/2);
printf( "sec.");
}
}
} while( buttons );
bbc_gcol(0, 7);
}
```

```
void scatter_list( int frame, int numactive )
```

```
{
int paths = 0;
int no_readings = 0;
int cell;
bbc_gcol( 0, 7 );
if( 60 / interval >= frame ) return;
for( cell = 0; cell < numactive; cell++ )
if( cellarray[60 / interval][cell].valid )
{
no_readings++;
paths += 0.36 *
(double)sqrt( sqr( cellarray[60/interval][cell].centre.x -
(double)cellarray[0][cell].centre.x)
+ (double)sqr( cellarray[60/interval][cell].centre.y - (double)cellarray[0][cell].centre.y)
);
}
```

Appendix I

```
}

void scatter( int frame, int numactive, char *name, char *details, char *date )
{
    char path [80];
    clrscr();
    cursor_off();
    pointer();
    text_colour(7);
    tab ( 58, 7);
    printf( "25µm/min." );
    tab (48, 7 );
    /* text_colour(6);
    printf( "100µm≤" ); */
    text_colour(7);
    tab( 31, 7);
    printf( "20µm");
    text_colour(7);
    tab( 65, 28 );
    printf( "%d", frame );
    printf( "*" );
    printf( "%d", interval);
    printf( "sec.");
    tab( 15, 20 );
    if( frame < 40)
    {
        printf( "%d", interval * 20 );
        printf( "sec.");
        tab( 42, 20 );
        printf( "%d", interval * 20 );
        printf( "sec.");
        tab(69, 20 );
        printf( "%d", interval * 20 );
        printf( "sec.");
    }
    if( frame > 40)
    {
        printf( "%d", interval * 40 );
        printf( "sec.");
        tab( 42, 20 );
        printf( "%d", interval * 40 );
        printf( "sec.");
        tab( 69, 20 );
        printf( "%d", interval * 40 );
        printf( "sec.");
    }
    tab( 4, 30 );
    text_colour(2); printf( "Details " );
    text_colour(7); printf( "%s", details );
    text_colour(2); tab( 4, 28 ); printf( "Title " );
    text_colour(7); printf( "%s", name );
    tab( 20, 28 );
    text_colour(5); printf( "%s", date );
    text_colour(2);
    scatter_list( frame, numactive );
    cell_pos_speed( frame, name, numactive);
    /*strcpy( path, "$plot." );
    strcat( path, name );
    sprite_screensave( path, 0 );*/
    pause();
}
```

Appendix 1

```
void graphs( int frame, int numactive, char *name, char *details, char *date )
{
    char path [80];
    int x_start = 200, y_start = 800, x_increment = 300, y_increment = -300;
    int length = 100;
    int x_start1, y_start1, paths, distance;
    int cell, fr;
    int x_drift, y_drift, sum_xvals, sum_yvals;
    int no_readings;
    int x, y, buttons;
    clrscr();
    /* Loop through every second frame */
    for( fr = 0; fr < frame; fr = fr + 2 )
    {
        /* plot a series of 12 axes */
        x_start1 = x_start + fr/2%4 * x_increment;
        y_start1 = y_start + fr/8%3 * y_increment;
        bbc_gcol( 0, 7 );
        bbc_move( x_start1 - length, y_start1 );
        bbc_draw( x_start1 + length, y_start1 );
        bbc_move( x_start1, y_start1 - length );
        bbc_draw( x_start1, y_start1 + length );
        /* calculate population drift for each frame */
        no_readings = 0, sum_xvals = 0, sum_yvals = 0, distance = 0;
        for( cell = 0; cell < numactive; cell++ )
            if( cellarray[fr][cell].valid )
            {
                no_readings++;
                sum_xvals += (cellarray[fr][cell].centre.x - cellarray[0][cell].centre.x);
                sum_yvals += (cellarray[fr][cell].centre.y - cellarray[0][cell].centre.y);
            }
        x_drift = sum_xvals/no_readings;
        y_drift = sum_yvals/no_readings;
        /* calculate the mean population displacement, distance, and plot as circle radius distance */
        no_readings = 0, paths = 0;
        for( cell = 0; cell < numactive; cell++ )
            if( cellarray[fr][cell].valid )
            {
                no_readings++;
                paths += (double)sqrt( sqr( cellarray[fr][cell].centre.x - (double)cellarray[0][cell].centre.x
- x_drift) +
                    (double)sqr( cellarray[fr][cell].centre.y - (double)cellarray[0][cell].centre.y -
y_drift) );
            }
        distance = paths/no_readings;
        bbc_gcol( 0, 7 );
        bbc_circle( x_start1, y_start1, distance * 3 );
        /* calculate the position of the cell centres and plot as circles radius 2 */
        for( cell = 0; cell < numactive; cell++ )
            if( cellarray[fr][cell].valid )
            {
                /* bbc_gcol( 0, 4 ); */
                bbc_gcol( 0, fr%7 + 1 );
                bbc_circlefill
                ( ( (double)cellarray[fr][cell].centre.x
- (double)cellarray[0][cell].centre.x - (double)x_drift) * 3 + x_start1,
                ( cellarray[fr][cell].centre.y
- cellarray[0][cell].centre.y - y_drift) * 3 + y_start1, 2 );
            }
    }
}
```

Appendix I

```
if((fr/2 + 1)%12 == 0)
```

```
{
    pause();
    strcpy( path, "$TRACK.jygraph." );
    strcat( path, name );
    sprite_screensave( path, 0 );
    clrscr();
}
do{
    mouse( &x, &y, &buttons );
} while( buttons );
}
pause();
}
```

```
void tracks( int frame, int numactive, char *name, char *details, char *date )
```

```
{
    char path [80];
    int x_start = 600, y_start = 500;
    int cell, fr;
    int x,y,buttons;
    clrscr();
    bbc_gcol( 0, 7 );
    bbc_move( x_start - 400, y_start );
    bbc_draw( x_start + 400, y_start );
    bbc_gcol(0, 7);
    bbc_circle( x_start, y_start, 60);
    bbc_move( x_start, y_start - 400 );
    bbc_draw( x_start, y_start + 400 );
    for( cell = 0; cell < numactive; cell++ )
    {
        for( fr = 1; fr < frame; fr++ )
        if( cellarray[fr][cell].valid )
        {
            bbc_gcol( 0, cell%7 + 1 );
            bbc_move(
                (( cellarray[fr - 1][cell].centre.x ) * 5 - (cellarray[0][cell].centre.x ) * 5 + x_start),
                (( cellarray[fr - 1][cell].centre.y ) * 5 - (cellarray[0][cell].centre.y ) * 5 + y_start));
            bbc_draw(
                (( cellarray[fr][cell].centre.x ) * 5 - (cellarray[0][cell].centre.x ) * 5 + x_start),
                (( cellarray[fr][cell].centre.y ) * 5 - (cellarray[0][cell].centre.y ) * 5 + y_start));
        }
    }
}
```

```
pause();
```

```
do
```

```
{
    mouse( &x, &y, &buttons );
    strcpy( path, "$trak." );
    strcat( path, name );
    sprite_screensave( path, 0 );
} while( buttons );
```

```
}
```

```
pause();
```

```
}
```

```
void trackdata( int frame, int numactive, char *name, char *details, char *date )
```

```
{
    FILE *file;
    int trakx_co = 0;
    int traky_co = 0;
    char path [80];
    int x_start = 600, y_start = 500;
    int cell, fr;
```

Appendix 1

```
clrscr();
bbc_gcol( 0, 7 );
bbc_move( x_start - 400, y_start );
bbc_draw( x_start + 400, y_start );
bbc_move( x_start, y_start - 400 );
bbc_draw( x_start, y_start + 400 );
strcpy( path, "$trakdata." );
strcat( path, name );
file = fopen( path, "w" );

for( fr = 1; fr < frame; fr++ )
{
    trakx_co = 0; traky_co = 0;
    for( cell = 0; cell < numactive; cell++ )
    if( cellarray[fr][cell].valid )
    {
        bbc_gcol( 0, cell%7 + 1 );
        bbc_move(
            (( cellarray[fr - 1][cell].centre.x ) * 5 - (cellarray[0][cell].centre.x ) * 5 + x_start),
            (( cellarray[fr - 1][cell].centre.y ) * 5 - (cellarray[0][cell].centre.y ) * 5 + y_start));
        bbc_draw(
            (( cellarray[fr][cell].centre.x ) * 5 - (cellarray[0][cell].centre.x ) * 5 + x_start),
            (( cellarray[fr][cell].centre.y ) * 5 - (cellarray[0][cell].centre.y ) * 5 + y_start));
        trakx_co = (cellarray[fr][cell].centre.x ) - (cellarray[0][cell].centre.x);
        traky_co = (cellarray[fr][cell].centre.y ) - (cellarray[0][cell].centre.y);
        fprintf(file, "%d,", trakx_co);
        fprintf(file, "%d,", traky_co);
    }
    else
    {
        fprintf(file, " , " );
        fprintf(file, " , " );
    }
    fprintf(file, "\n" );
} fclose( file );
```

```
pause();
}
```

```
void histograms( int frame, int numactive, char *name, char *details, char *date )
/* Takes cell displacements - drift grouped in predetermined band width listed in counter and
finds
```

```
the number of cells whose displacements fall in that band width ie size[counter] */
```

```
{
    char path [80];
    int x_start = 40, y_start = 720, x_increment = 360, y_increment = -360;
    int length = 320, x_start1, y_start1;
    int cell, fr;
    int size[100];
    int counter;
    int x_drift, y_drift, sum_xvals, sum_yvals;
    int no_readings;
    int x, y, buttons;
    clrscr();
    for( fr = 0; fr < frame; fr = fr + 2 )
    {
        x_start1 = x_start + fr/2%3 * x_increment;
        y_start1 = y_start + fr/6%3 * y_increment;
        bbc_gcol( 0, 7 );
        bbc_move( x_start1, y_start1 );
        bbc_draw( x_start1 + length, y_start1 );
```


Appendix I

```
bbc_move( x_start1, y_start1 );
bbc_draw( x_start1, y_start1 + length );
for(counter = 0; counter < 100; counter++)
{
    size[counter] = 0;
}
sum_xvals = 0; sum_yvals = 0;
for( cell = 0; cell < numactive; cell++ )
if( cellarray[fr][cell].valid )
{
    no_readings++;
    sum_xvals += (cellarray[fr][cell].centre.x - cellarray[0][cell].centre.x);
    sum_yvals += (cellarray[fr][cell].centre.y - cellarray[0][cell].centre.y);
}
x_drift = sum_xvals/no_readings;
y_drift = sum_yvals/no_readings;
for( cell = 0; cell < numactive; cell++ )
if( cellarray[fr][cell].valid )
{
    counter = (double)sqrt( sqrt( cellarray[fr][cell].centre.x
        - (double)cellarray[0][cell].centre.x
        - (double)x_drift ) + (double)sqrt( cellarray[fr][cell].centre.y
        - (double)cellarray[0][cell].centre.y - (double)y_drift )/4 );
    size[counter] = (size[counter] + 1);
}
bbc_gcol( 0, 6);
for(counter = 0; counter < 60; counter++)
{
    bbc_rectanglefill(x_start1 + 4 * counter, y_start1, 4, 20 * size[counter]);
}
if((fr/2 + 1)%9 == 0)
{
    pause();
    strcpy( path, "$.jyhist." );
    strcat( path, name );
    sprite_screensave( path, 0 );
    clrscr();
}
do{
    mouse( &x, &y, &buttons );
} while( buttons );
}
pause();
}
void root( int frame, int numactive )
{
    int x_start = 200, y_start = 100;
    int cell, fr, no_readings, x_drift, y_drift;
    int time_span, paths;
    int sum_xvals = 0, sum_yvals = 0;
    int x0, y0, x1, y1;
    int x, y, buttons;
    clrscr();
    bbc_gcol( 0, 7 );
    bbc_move( x_start - 200, y_start );
    bbc_draw( x_start + 800, y_start );
    bbc_move( x_start, y_start );
    bbc_draw( x_start, y_start + 800 );
    bbc_gcol( 0, 6 );
    for( fr = 1; fr < frame; fr++ )
    {
```

Appendix I

```
time_span = FACTOR / interval;
no_readings = 0; sum_xvals = 0; sum_yvals = 0;
for( cell = 0; cell < numactive; cell++ )
if( cellarray[fr][cell].valid )
{
    no_readings++;
    sum_xvals += (cellarray[fr][cell].centre.x - cellarray[fr-1][cell].centre.x);
    sum_yvals += (cellarray[fr][cell].centre.y - cellarray[fr-1][cell].centre.y);
}
x_drift = sum_xvals/no_readings;
y_drift = sum_yvals/no_readings;
paths = 0, no_readings = 0;
for( cell = 0; cell < numactive; cell++ )
if( cellarray[fr][cell].valid )
{
    no_readings++;
    paths += time_span *
        (double)sqrt( sqr( cellarray[fr][cell].centre.x
            - (double)cellarray[0][cell].centre.x - x_drift)
        + (double)sqr( cellarray[fr][cell].centre.y
            - (double)cellarray[0][cell].centre.y - y_drift) );
}
bbc_circle(x_start + 400/fr, y_start + 100000/(sqr(paths/no_readings)), 4);
}
pause();
bbc_gcol(3, 7);
mouse( &x, &y, &buttons );
x0 = x; y0 = y;
mouse( &x, &y, &buttons );
bbc_move(x0, y0);
bbc_draw( x, y );
x1 = x; y1 = y;
do{
    mouse( &x, &y, &buttons );
    if( x != x1 || y != y1 )
    {
        bbc_move(x0, y0);
        bbc_draw( x1, y1 );
        bbc_move(x0, y0);
        bbc_draw( x, y );
        x1 = x; y1 = y;
        if( x0 != x1 )
        {
            tab(18, 20);
            printf( "      ");
            tab(18, 20);
            printf( "%d", 10000 * (y0 - y1)/(x0 - x1)/100);
        }
        if(y0 != y1 )
        {
            tab(18, 21);
            printf( "      ");
            tab(18, 21);
            printf( "%d", x0 - x_start - (x0 - x1) * (y0 - y_start) / (y0 - y1));
        }
    }
} while( buttons );
pause();
}
```

Appendix I

```
void ratio_plot( int frame, int numactive )
{
    float mean_speed = 0, mean_ratio = 0;
    int y_start = 200, x_start = 200;
    float ratio = 0, speed = 0;
    int cell, fr;
    for(fr = 1; fr < frame; fr++)
    {
        speed = 0; ratio = 0;
        for( cell = 0; cell < numactive; cell++)
        if( cellarray[fr][cell].valid )
        {
            speed += (float)(sqrt(fsqr(cellarray[fr][cell].centre.x - cellarray[fr-1][cell].centre.x) +
                                fsqr(cellarray[fr][cell].centre.y - cellarray[fr-1][cell].centre.y)))/5;
            ratio += (float)cellarray[fr][cell].area/cellarray[fr][cell].perimeter;
        }
        /*printf("area %d, perimeter %d\n",cellarray[fr][cell].area,cellarray[fr][cell].perimeter);
        printf("ratio %4.2f, sp %4.2f\n",ratio,speed);*/
        bbc_gcol( 0, 7 );
        bbc_move( 200,200 );
        bbc_draw( 200,1000 );
        bbc_move( 200,200 );
        bbc_draw( 1000,200 );
        bbc_gcol( 0, cell%7 + 1 );
        bbc_circlefill(x_start + ( 100*ratio ), y_start + (100 * speed),2 );
        pause();
    }
}

void display( int frame, int numactive, char *name, char *details, char *date )
{
    int quit = 0;
    int choice;
    int x, y, buttons;

    do{
        clrscr();
        cursor_off();
        pointer();

        tab( 5, 2 );
        text_colour( 7 );
        printf( "1: Scatter." );
        tab( 5, 4 );
        printf( "2: Square-root." );
        tab( 5, 6 );
        printf( "3: Graphs." );
        tab( 5, 8 );
        printf( "4: Histograms." );
        tab( 5, 10 );
        printf( "5: Tracks" );
        tab( 5, 12 );
        printf( "6: Trackdata" );
        tab( 5, 14 );
        printf( "7: Ratio" );
        tab( 5, 16 );
        printf( "8: Quit" );
        do{
```

Appendix I

```
    mouse( &x, &y, &buttons );
} while( buttons );

do{
    choice = keypressed();
    if( choice != -1 )
        choice -= '0';
    else
        choice = 0;

    mouse( &x, &y, &buttons );
    if( buttons == 4 )
        choice = 15 - y / 64;
} while( choice == 0 );

switch( choice )
{
    case 1 :
        scatter( frame, numactive, name, details, date );
        break;

    case 2 :
        root( frame, numactive);
        break;

    case 3 :
        graphs( frame, numactive, name, details, date );
        break;

    case 4 :
        histograms( frame, numactive, name, details, date );
        break;

    case 5 :
        tracks( frame, numactive, name, details, date );
        break;

    case 6 :
        trackdata( frame, numactive, name, details, date );
        break;

    case 7 :
        ratio_plot( frame, numactive );
        break;

    case 8 :
        quit = 1;
        break;

}
} while( quit == 0 );
}
```

Appendix II

Program written in C, to run on an Acorn Archimedes microcomputer; to collect data from the LKB Luminometer

Program plots the results on screen, and calculates the area under the curve for each sample.

```
#include<stdlib.h>
#include<stdio.h>
#include<bbc.h>
#include<math.h>
#include<ctype.h>
#include<os.h>
#include<string.h>
#include<time.h>
#define maxtubes 15
#define maxloops 180
typedef struct
{
    float tot_area;
    float reading;
    float time_sec;
    float area;
} tube_data;

/* global variables */
tube_data tube_array[maxloops][maxtubes];
char name[20], details[200], date[20];
int no_loops = 0;
float scaler = 1.0;
int no_tubes = 0;
int stim = 0;
int main(void);
int plot_results(int lp, int tb);
int store_results(int no_loops);
void display_results(void);
void stop_luminometer(void);
int rescale(int lp, int tb);
/* emulate *fx a,x and define some basic routines
definitions of fx() function calls:
fx( 2, 1 )- gets characters from RS423 port.
fx( 2, 0 )-gets characters from the keyboard.
fx( 3, -selects output stream direction:
fx( 3, 0 )-printer, screen
fx( 3, 1 )-printer, screen, RS423
fx( 3, 2 )-printer
fx( 3, 3 )-printer,      RS423
fx( 3, 4 )-      screen
fx( 3, 5 )-      screen, RS423
fx( 3, 6 )-
fx( 3, 7 )-      RS423
fx( 7, 3 )- receiving RS423 baud rate of 300(luminometer)
fx( 8, 3 )- transmit RS423 baud rate of 300(luminometer)
fx( 229, 1 )-sets escape key to produce ascii character &1B or 27
fx( 229, 0 )-resets escape key to interrupt program*/

void fx( int a, int x )
{
    int y;
    os_byte( a, &x, &y );
}
```

Appendix II

```
int keypressed()
{
    return bbc_inkey( 0 );
}

void mouse( int *x, int *y, int *buttons )
{
    os_regset regs;
    os_swi( 0x1c, &regs );
    *x = regs.r[0];
    *y = regs.r[1];
    *buttons = regs.r[2];
}

void pause()
{
    int flag = 0;
    int x, y, buttons;

    do{
        mouse( &x, &y, &buttons );
    } while( buttons );

    do{
        if( keypressed() != -1 ) flag = 1;
        mouse( &x, &y, &buttons );
        if( buttons ) flag = 1;
    } while( !flag );
}

void cat()
{
    os_cli( "cat $.LUMIN.result" );
}

void pointer( void )
{
    int x = 1, y;

    os_byte( 0x6a, &x, &y );
}

/* asks user for number of tubes to be measured*/
void tube_entry()
{
    int flag = 1;
    no_tubes = 0;
    fx( 2, 0 );
    while( flag == 1 )
    {
        bbc_tab( 20, 20);
        printf( "Enter the number of tubes to be assayed (1-25)\n" );
        bbc_tab(20, 22);
        printf("      ");
        bbc_tab(20, 22);
        scanf( "%d", &no_tubes );
        if( no_tubes > 0 && no_tubes < 25 )
        {
            flag = 0;
        }
    }
    return;
}
```

Appendix II

```
/* asks user which stimulant of metabolic burst is being used */
void loop_entry()
{
    int timerun = 0;
    int flag = 1;
    fx(2,0);
    while( flag == 1 )
    {
        bbc_tab( 20, 25 );
        printf(" ");
        bbc_tab( 20, 24 );
        printf("Enter the time for run in minutes ( 1 - 100 )");
        bbc_tab( 20, 25 );
        scanf(" %d", &timerun );
        no_loops = ( timerun * 60 ) / (( no_tubes * 2 ) + 15 );
        if( timerun > 0 && timerun < 100 )
        {
            flag = 0;
        }
    }
    return;
}
/* enters luminometer to auto-mode*/
void auto_operation()
{
    int num = 0;
    int bitmask = 127;
    int inchar;
    int in_char;
    bbc_cls();
    bbc_tab( 20, 18 );
    printf(" To enter luminometer to automode:");
    bbc_tab( 20, 20 );
    printf("1. Switch off luminometer.");
    bbc_tab( 20, 22 );
    printf("2. Restart luminometer whilst holding down");
    bbc_tab( 22, 24 );
    printf(" preset and store buttons simultancously.");
    bbc_tab( 20, 26 );
    printf("3. Press return");
    bbc_tab( 20, 28 );
    printf("4. 'TYPE' contrIQSP1 ");
    pause();
    fx( 156, 16 );
    fx( 21, 0 );
    fx( 5, 1 );
    bbc_cls();
    bbc_mode(3);
    bbc_vdu(3);
    bbc_vdu(28);
    bbc_vdu(4);
    bbc_vdu(22);
    bbc_vdu(76);
    bbc_vdu(5);
    bbc_cursor(1);
    fx( 7, 3 );
    fx( 8, 3 );
    fx( 6, 0 );
    fx( 3, 5 );
    fx( 2, 0 );
    fx( 229, 1 );
}
```

Appendix II

```
in_char = getchar();
printf("%c", in_char );
fx( 2, 1 );
while( inchar != 33)
{
    in_char = getchar();
    inchar = in_char & bitmask;
    printf("%c", inchar );
    if ( in_char == 10){num++;}
    if ( num == 3){inchar = 33;}
}
fx( 229, 0 );
fx( 3, 0 );
fx( 2, 0 );
bbc_mode(21);
return;
}

/* draws the axes of the graphs for plotting results*/
void experiment()
{
    int colour_plot[18] = { 67, 75, 76, 19, 27, 119,
                           19, 56, 95, 78, 60, 123,
                           23, 7, 31, 30, 44, 107 };

    int i;
    int ctr = 0;
    int y_val = 0;
    bbc_cursor(0);
    bbc_gcol( 0, 47);
    bbc_move(10, 100);
    bbc_draw(10, 900);
    bbc_move(10, 100);
    bbc_draw(900, 100);
    for( i = 1; i < 9; i++ )
    {
        bbc_gcol( 0, 47);
        bbc_move( 100*i, 90);
        bbc_draw( 100*i, 100);
        bbc_move( 0, 100 + 100*i);
        bbc_draw( 10, 100 + 100*i);
    }
    bbc_colour( 77 );
    bbc_tab( 30, 2 );
    printf( "LKB LUMINOMETER");
    y_val = 6;
    for( ctr = 0; ctr < no_tubes; ctr++ )
    {
        y_val++;
        bbc_colour(50);
        bbc_gcol(0, colour_plot[ctr]);
        bbc_tab( 44, y_val );
        printf(" tube num %d", ctr + 1 );
    }
}

/* takes the results from the luminometer and converts
   them into decimal values*/
void input_results()
{
    int flag = 0;
    int tb = 0; int lp = 0;
    float sum_reading = 0;
```


Appendix II

```
int tubenum = 0;
int tube[4] = 0;
int reading[8] = 0;
int num;
int in_char;
int inchar;
int bitmask = 127;
float time_elapsed = 0;
int ctr = 0;
for(tb= 0; tb<no_tubes; tb++)
{
    tube_array[0][tb].tot_area = 0;
}
fx( 3, 6 );
fx( 2, 1 );
fx( 7, 3 );
fx( 15, 0 );
fx( 229, 1 );
bbc_cursor(0);
bbc_tab( 0, 4 );
while(bbc_adval(-2) == 0 )
{
    time_elapsed = 0;
    while (inchar != 89)
    {
        fx ( 2, 1 );
        in_char = getchar();
        for( lp = 0; lp < no_loops; lp++ )
        {
            bbc_tab( 43, 6 + no_tubes );
            printf(" ");
            time_elapsed = time_elapsed + 2 + no_tubes;
            for( tb = 0; tb < no_tubes; tb++ )
            {
                fx( 2, 0 );
                if ( keypressed() == 's')
                {
                    no_loops = lp;
                    stop_luminometer();
                    store_results(no_loops);
                }
            }
            fx( 3, 6 );
            fx( 2, 1 );
            num = 0; tubenum = 0;
            in_char = getchar();
            inchar = in_char & bitmask;
            while( inchar != 13 )
            {
                tube[num] = inchar - 48;
                in_char = getchar();
                inchar = in_char & bitmask;
                num++;
            }
            tubenum = (tube[2] * 10) + tube[3];
            num = 0; tube[num] = 0;
            in_char = getchar();
            inchar = in_char & bitmask;
            while( inchar != 13)
            {
                reading[num] = inchar - 48;
                in_char = getchar();
```

Appendix II

```
    inchar = in_char & bitmask;
    num++;
}
sum_reading = 0;
if( reading[1] == -16 && reading[2] == -16 && reading[3] == -16 )
{
    sum_reading = (float)reading[4] + (float)reading[6]/10 +
        (float)reading[7]/100 + (float)reading[8]/1000;
    flag = 1;
}
if ( reading[1] == -16 && reading[2] == -16 && reading[3] != -16 )
{
    sum_reading = ((float)reading[3] * 10) + (float)reading[4] +
        (float)(reading[6] / 10.0) + (float)(reading[7] / 100.0);
}
if( reading[1] == -16 && reading[2] != -16 && reading[3] != -16 )
{
    sum_reading = ((float)reading[2] * 100.0) + (float)(reading[3] * 10.0) +
        (float)reading[4] + (float)(reading[6] / 10.0);
}
if( reading[1] == -16 && reading[2] == -16 && reading[3] == -16 && reading[5] != 46
&& flag!=1)
{
    for(ctr = 1000; ctr == 1; ctr / 10)
    {
        sum_reading += (float)(reading[4] * 1000) +
            (float)(reading[5] * 100) +
            (float)(reading[6] * 10) +
            (float)(reading[7] * 1);
    }
    flag = 0;
}
time_elapsed = time_elapsed + 1;
tube_array[lp][tb].reading = sum_reading;
tube_array[lp][tb].time_sec = time_elapsed;
if(sum_reading > 1000){rescale( lp, tb);}
plot_results(lp, tb);
}
}
fx(3,0);
fx( 2, 0 );
fx( 7, 7 );
fx( 8, 7 );
store_results(no_loops);
exit(1);
}
return;
}

/*plots the data from the luminometer*/
int plot_results(int lp, int tb)
{
    float time_dash = 0;
    float dash = 0;
    int y_val;
    float x_start = 10.0;
    float y_start = 100.0;
    int colour_plot[18] = { 67, 75, 76, 19, 27, 119,
        19, 56, 95, 78, 60, 123,
        23, 7, 31, 30, 44, 107 };
}
```

Appendix II

```
float triang_area = 0;
float rectang_area = 0;
fx( 3, 0 );
fx( 2, 0 );
bbc_colour(57);
bbc_tab( 1,2 );
printf("Loop %d of %d ", lp+1, no_loops );
bbc_tab( 1, 4 );
printf("-----");
bbc_colour( colour_plot[tb] );
y_val = 7 + tb - 1;
bbc_tab( 43, y_val );
printf(" ");
y_val = 7 + tb;
bbc_tab( 43, y_val );
printf(">");
bbc_gcol(0,colour_plot[tb]);
dash = (( y_start + tube_array[lp][tb].reading / scaler ) - (y_start + tube_array[lp-1][tb].reading / scaler )) / 2.0;
time_dash = ( tube_array[lp][tb].time_sec - tube_array[lp-1][tb].time_sec ) / 2;
if( lp > 0 )
{
    if( tb%2 == 0 )
    {
        bbc_move( x_start + tube_array[lp-1][tb].time_sec,
                  y_start + tube_array[lp-1][tb].reading / scaler );
        bbc_draw( x_start + tube_array[lp][tb].time_sec,
                  y_start + tube_array[lp][tb].reading / scaler );
    }
    else
    {
        bbc_move( x_start + tube_array[lp-1][tb].time_sec,
                  y_start + tube_array[lp-1][tb].reading / scaler );
        bbc_draw( x_start + tube_array[lp-1][tb].time_sec + time_dash,
                  y_start + (tube_array[lp-1][tb].reading / scaler) + dash );
        bbc_gcol(0, 63 );
        bbc_move( x_start + tube_array[lp-1][tb].time_sec + time_dash,
                  y_start + (tube_array[lp-1][tb].reading / scaler) + dash );
        bbc_draw( x_start + tube_array[lp][tb].time_sec,
                  y_start + tube_array[lp][tb].reading / scaler );
        bbc_gcol( 0, colour_plot[tb] );
    }
}
triang_area = 0.5 * (( tube_array[lp][tb].time_sec - tube_array[lp-1][tb].time_sec ) *
                    ( tube_array[lp][tb].reading - tube_array[lp-1][tb].reading ));
rectang_area = ( tube_array[lp][tb].time_sec - tube_array[lp-1][tb].time_sec ) *
                tube_array[lp-1][tb].reading;
tube_array[lp][tb].area = triang_area + rectang_area;
tube_array[0][tb].tot_area += tube_array[lp][tb].area;
bbc_rectanglefill( 900, 780 - tb*32,tube_array[0][tb].tot_area/500 , 15 );
return no_loops;
}

/*creates file called lumi_prog which contains the program to run the luminometer*/
void lumi_prog( )
{
    FILE *fp;
    fx( 2,0 );
    fx( 3,4 );
    fp = fopen( "Lumi_prog", "w" );
    fputs( "50 CONTIMIX\n", fp );
}
```

Appendix II

```
fprintf(fp, "90 LOOP %d \n", no_loops );
fputs( "100 FIRSTIN \n", fp );
fprintf( fp, "110 LOOP %d\n", no_tubes );
fputs( "120 PRPOS \n", fp );
fputs( "125 PRC \n", fp );
fputs( "140 NEXT \n", fp );
fputs( "150 ENDLOOP \n", fp );
fputs( "160 WAIT 0 \n", fp );
fputs( "170 ENDLOOP \n", fp );
fputs( "280 OFFMIX \n", fp );
fputs( "300 OUT \n", fp );
fputs( "RUN\n", fp );
fclose(fp);
}

/* writes the program from lumi_prog through the rs423 port*/
void send_to_lumi( )
{
    int in_char;
    int ctr;
    FILE *fp;
    fp = fopen( "Lumi_prog", "r" );
    fx( 7, 3 );
    fx( 8, 3 );
    fx( 2, 1 );
    fx( 3, 5 );

    in_char = fgetc( fp );
    while ( in_char != EOF )
    {
        printf("%c", in_char);
        in_char = fgetc( fp );
    }
    fclose(fp);
    fx( 2, 0 );
    fx(3,0);
    bbc_tab( 20, 43 );
    printf(" wait ");
    for ( ctr = 0 ; ctr < 4000000; ctr++){ }
    return;
}

/* request date details and filename from user and then create file called filename
with the dsta*/
int store_results(int no_loops)
{
    FILE *file;
    char path[80];

    fx( 3, 0 );
    fx( 2, 0 );
    fx( 229, 0 );
    bbc_cls();
    bbc_mode(21);
    do{
        bbc_tab( 2, 2 );
        printf( "Enter date:- " );
        gets( date );
        bbc_tab( 2, 4 );
        printf( "Enter details:- " );
        gets( details );
    }
```

```
    bbc_tab ( 2,6 );
    printf( "ENTER FILE NAME " );
    gets( name );
    strcpy( path, "S.LUMIN.result." );
    strcat( path, name );
    file = fopen( path, "w" );
    if( file == NULL ) printf( "CANNOT OPEN FILE" );
} while( file == NULL );
fwrite( name, sizeof( char ), 20, file );
fwrite( details, sizeof( char ), 160, file );
fwrite( date, sizeof( char ), 20, file );
fwrite( &no_loops, sizeof( int ), 1, file );
fwrite( &no_tubes, sizeof( int ), 1, file );
fwrite( tube_array, sizeof( tube_data ), maxloops * maxtubes, file );
fclose( file );
display_results();
return no_loops;
}

/* loads file entered by user and displays results*/
void load()
{
    int ch = 0;
    char path[80];
    FILE * file;
    int error;
    bbc_mode(21);
    bbc_tab( 2, 4 );
    printf( "Avaliable files:\n" );
    cat();
    printf("&d",ch);
    do{
        printf( "\nEnter name of desired file: " );
        gets( name );
        strcpy( path, "S.LUMIN.result." );
        strcat( path, name );
        file = fopen( path, "r" );
        error = 0;
        if( file == NULL )
        {
            printf( "Cannot open file.\n" );
            error = 1;
        }
    } while( error == 1 );
    fread( name, sizeof( char ), 20, file );
    fread( details, sizeof( char ), 160, file );
    fread( date, sizeof( char ), 20, file );
    fread( &no_loops, sizeof( int ), 1, file );
    fread( &no_tubes, sizeof( int ), 1, file );
    fread( tube_array, sizeof( tube_data ), maxloops * maxtubes, file );
    display_results();
    fclose( file );
    pause();
    return;
}

/* displays results*/
void display_results(void)
{
    FILE *fp;
    int time_dash = 0;
    int dash = 0;
```

Appendix II

```
int x_start = 10;
int y_start = 100;
int colour_plot[18] = { 67, 75, 76, 19, 27, 119,
                        19, 56, 95, 78, 60, 123,
                        23, 7, 31, 30, 44, 107 };

int tb = 0;
int lp = 0;
int incr = 10;
int num = 0;
int y_val = 8;
char path[80];
bbc_cls();
experiment();
fx( 2, 0 );
fx( 3, 0 );
fx( 7, 7 );
fx( 8, 7 );
fx ( 5, 0 );
bbc_tab( 2, 4 );
printf("File: %s", name);
bbc_tab( 24, 4 );
printf(" Details: %s", details);
strcpy( path, "$LUMIN.csv." );
strcat( path, name);
fp = fopen( path, "w" );
for( lp = 0; lp < no_loops; lp++ )
{
    for( tb= 0; tb < no_tubes; tb++ )
    {
        fprintf(fp, "%f", tube_array[lp][tb].time_sec );
        fprintf(fp, "%f", tube_array[lp][tb].reading );
        if( tube_array[lp][tb].reading > 1000){rescale( lp, tb);}
        bbc_gcol(0, colour_plot[tb]);
        bbc_rectanglefill( 900, 780 - tb*16, tube_array[0][tb].tot_area/500, 10 );
        dash = (( y_start + tube_array[lp][tb].reading / scaler )
                - ( y_start + tube_array[lp-1][tb].reading / scaler )) / 2;
        time_dash = ( tube_array[lp][tb].time_sec - tube_array[lp-1][tb].time_sec ) / 2;
        if( lp > 0 )
        {
            if( tb%2 == 0 )
            {
                bbc_move( x_start + tube_array[lp-1][tb].time_sec,
                          y_start + tube_array[lp-1][tb].reading / scaler );
                bbc_draw( x_start + tube_array[lp][tb].time_sec,
                          y_start + tube_array[lp][tb].reading / scaler );
            }
            else
            {
                bbc_move( x_start + tube_array[lp-1][tb].time_sec,
                          y_start + tube_array[lp-1][tb].reading / scaler );
                bbc_draw( x_start + tube_array[lp-1][tb].time_sec + time_dash,
                          y_start + (tube_array[lp-1][tb].reading / scaler) + dash );
                bbc_gcol( 0, 63 );
                bbc_move( x_start + tube_array[lp-1][tb].time_sec + time_dash,
                          y_start + (tube_array[lp-1][tb].reading / scaler) + dash );
                bbc_draw( x_start + tube_array[lp][tb].time_sec,
                          y_start + tube_array[lp][tb].reading / scaler );
            }
        }
    }
}
fprintf( fp, "\n");
```

Appendix II

```
}
num = 0;
printf("\n");
for( tb= 0; tb < no_tubes; tb++ )
{
    num++;
    bbc_tab( incr+40, y_val + num + 10);
    printf("Total area for tube %d: %4.0f\n",tb + 1,tube_array[0][tb].tot_area);
}
fclose(fp);
fx( 2, 0 );
fx( 5, 0 );
fx( 3, 0 );
fx( 7, 7 );
fx( 8, 8 );
pause();
main();
return;
}
```

```
int rescale(int lp, int tb)
{
    int x_start = 10;
    int y_start = 100;
    int ctr = 0;
    scaler = abs(tube_array[lp-1][tb].reading/1000)+1;
    for( ctr = 0; ctr < lp; ctr++ )
    {
        for( tb = 0; tb < no_tubes; tb++ )
        {
            experiment();
            if( ctr > 0 )
            {
                bbc_move( x_start + tube_array[lp-1][tb].time_sec,
                           y_start + tube_array[lp-1][tb].reading / scaler );
                bbc_draw( x_start + tube_array[lp][tb].time_sec,
                           y_start + tube_array[lp][tb].reading / scaler );
            }
        }
    }
    return no_loops;
}
```

/* sends the character for escape to the luminometer*/

void stop_luminometer(void)

```
{
    fx( 21, 1 );
    fx( 21, 2 );
    fx( 21, 3 );
    fx( 156, 16 );
    fx( 229, 1 );
    fx( 7, 3 );
    fx( 8, 3 );
    fx( 5, 2 );
    bbc_vdu(2);
    bbc_vduq( 1, 16);
    bbc_vdu(3);
    fx( 3, 0 );
    fx( 2, 0 );
    fx( 229, 0 );
    return;
}
```

Appendix II

```
}

/* The programme menu */

int main(void)
{
    int quit = 0;
    int choice;
    int x, y, buttons;
    bbc_mode( 21 );
    do{
        bbc_cls();
        pointer();
        bbc_tab( 5, 0 );
        printf( " I.KB LUMINOMETER MENU" );
        bbc_tab( 5, 2 );
        printf( "1: RUN" );
        bbc_tab( 5, 4 );
        printf( "2: LOAD FILE " );
        bbc_tab( 5, 6 );
        printf( "3: DISPLAY RESULTS" );
        bbc_tab( 5, 8 );
        printf( "4: AUTO OPERATION" );
        bbc_tab( 5, 10 );
        printf( "5: STOP LUMINOMETER" );
        bbc_tab( 5, 12 );
        printf( "6 : QUIT" );
        do{
            mouse( &x, &y, &buttons );
        }while( buttons );
        do{
            choice = keypressed();
            if( choice != -1 )
                choice -= '0';
            else
                choice = 0;
            mouse( &x, &y, &buttons );
            if( buttons == 4 )
                choice = 15 - y / 64 ;
        }while( choice == 0 );
        switch( choice )
        {
            case 1 :
                bbc_cls();
                tube_entry();
                loop_entry();
                lumi_prog();
                send_to_lumi();
                bbc_cls();
                experiment();
                input_results();
                break;

            case 2 :
                bbc_cls();
                bbc_tab( 20, 8 );
                load();
                break;

            case 3 :
                bbc_cls();
```


Appendix II

```
    display_results();
    pause();
    break;
case 4 :
    bbc_cls();
    auto_operation();
    break;
case 5 :
    bbc_cls();
    stop_luminometer();
    break;
case 6 :
    bbc_cls();
    exit(1);
    fx( 2, 0 );
}
} while( quit == 0 );
}

/*Header File*/
/* Functions from screen */
void plot( point p );
void move( point p );
void draw ( point p );
void graphics_colour( int c );
void gccl( int c );
void mode( int m );
void cursor_off( void );
void clrscr( void );
void text_colour( int );
void tab( int x, int y );
char get( void );
int keypressed( void );
void palette( char a, char b, char c, char d );
void settxtwindow( void );
void clearwindow( void );

/* Functions from basic */
int MAX( int a, int b );
int MIN( int a, int b );
int sqr( int );
float fsqr( float );
void pause( void );
```